Modulation of UVA induced HO-1 gene expression by carotenoids in cultured fibroblasts

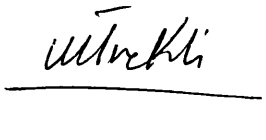
Trekli, Marika

Award date:
2003

Awarding institution:
University of Bath

MODULATION OF UVA INDUCED HO-1 GENE EXPRESSION BY CAROTENOIDS IN CULTURED FIBROBLASTS

Submitted by Marika Trekli
for the degree of PhD
of the University of Bath
2003



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Summary

The human heme oxygenase 1 (HO-1) gene is strongly activated following UVA irradiation of normal human dermal fibroblasts (FEK4) and this response is being used as a marker of oxidative stress in cells. Evidence suggests that the activation of HO-1 gene by UVA radiation occurs via a singlet oxygen ($^1O_2$) mediated pathway. Carotenoids are potent $^1O_2$ quenchers and are expected to effectively suppress the UVA induced HO-1 gene expression. However, approximately 10% of carotenoids also function as vitamin A precursors and it is possible that the biological activity of carotenoids might be due to retinoid signalling. We used a provitamin A carotenoid (β-carotene) and a non-provitamin A carotenoid (lycopene) for studying the modulation of the UVA induced HO-1 mRNA levels by carotenoids.

This is the first study to show a concentration-dependent suppression of the UVA (250 kJ/m^2) induced of HO-1 mRNA levels after incubation of FEK4 cells with a series of six all-trans-β-carotene concentrations (0.07, 0.2, 0.8, 2.3, 8.0, 21 μM) in the culture medium and a contrasting lack of suppression of the UVA (100 kJ/m^2) induced HO-1 mRNA levels following incubation with β-carotene at all the above concentrations, except for the highest (21 μM).

This is also the first study to show a suppression of UVA (100 kJ/m^2) induction of HO-1 mRNA levels after incubation of FEK4 cells with a series of five lycopene concentrations in the culture medium. The UVA (250 kJ/m^2) induced HO-1 levels were suppressed following incubation of FEK4 cells with lycopene at the lowest concentrations (0.1, 0.3 and 1.2 μM) tested. The corresponding levels of β-carotene and lycopene uptake by FEK4 cells, as well as isomerisation and apo-carotenal formation, were measured following HPLC separation. The findings of this study support the hypothesis of singlet oxygen quenching as the mode of action of lycopene for reducing UVA mediated oxidative stress, but the possibility that β-carotene exerts its effects through retinoid signalling could also be considered.

PUBLICATION

Trekli, M.; Riss, G.; Goralczyk, R.; Tyrrell, R.M. Beta carotene suppresses UVA-induced HO-1 gene expression in cultured FEK4. *Free Rad. Biol. Med.* 34(4): 456-464; 2003.

CONTENTS

1 INTRODUCTION

1.1 General aspects

Solar radiation has long been known to have beneficial as well as deleterious effects on humans. Beneficial effects of solar UV include an essential role in the production of vitamin D3 as well as regulation of vitamin D metabolism. This vitamin is essential for maintaining blood calcium levels within a normal range and therefore for the growth and maintenance of a healthy skeleton. Hallmarks of therapeutic advances have been the introduction of artificial ultraviolet lamps and sunlight for the treatment of skin tuberculosis by Finsen in 1900 and of photochemotherapy with oral psoralen and long wave UV in by Parish in 1974. Ultraviolet and visible light alone (phototherapy) or in combination with chemical agents (photochemotherapy, photodynamic therapy) are now used in a variety of medical interventions. These include the treatment of hyperbilirubinemia in the newborn, treatment of psoriasis, atopic dermatitis and photodynamic therapy for tumors. However, strong evidence has been accumulated in the last decades implicating solar UV in various physiological and pathological states. These include erythema, sunburn pigmentation hyperplasia, photodermatosis, premature skin aging, immunosuppression, activation of infections, cataract formation, melanoma- and non-melanoma-type skin cancers (for reviews see: Applegate and Frenk, 1995; Tyrrell, 1994; 1996; 1997; Pourzand *et al.,* 2000; Termorshuizena, 2002).

Developing strategies for protection against the adverse effects of solar radiation is a desirable goal. The means for protection against UV in humans can be divided into two categories: topical and systemic protection. Research is involved in the development of sunscreen formulations that protect against UV induced erythema, immunosuppression (Fourtanier *et al.*, 2000) and DNA damage related to skin cancer (Young *et al.*, 2000). Recently, the use of retinoic acid has been proposed for preventing functional vitamin A deficiency in human skin by solar radiation (Wang *et al.*, 1999a). No systemic drugs are generally recommended for protection of normal human skin against solar exposure. High doses of β-carotene are effective in reducing photosensitivity in patients with erythropoietic protoporphyria (Mathews-Roth, 1993). β-carotene when administered intraperitoneally has been shown to inhibit UV induced epidermal damage and tumor formation in a mouse model (Epstein, 1977). Epidemiological evidence suggests an inverse relationship between high levels of circulating carotenoids and cancer incidence in humans (for review see Mayne, 1996; Peto *et al.*, 1981; Block *et al.*, 1992; Ziegler, 1989). However, Greenberg and colleagues (Greenberg *et al.*, 1990) demonstrated that supplementation with β-carotene did not reduce the occurrence of new skin cancers in patients with a previous history of non-melanoma skin cancer.

In this thesis we initially gather together lines of evidence regarding the effects of the UVA component of sunlight on cells in culture and on the pathways that mediate these effects (section 1.2). The next part of the introduction is concerned with possible physiological roles of the HO gene and the inducible HO-1 isoform (section 1.3). The chemical and biological properties of carotenoids, as well as the results of epidemiological studies and intervention trials are summarised in section 1.4. As carotenoid metabolites have been shown to interact with nuclear receptors that are major transcriptional regulators, a main concern in the literature is whether the actions of carotenoids in cells and tissues are intrinsic to carotenoids or due to activation of nuclear receptors. Section 1.5 is a brief overview of the nuclear receptors especially of those likely to interact with the experimental results. The particularities of carotenoid work are presented in the final part of the introduction (section 1.6).

1.2 The UV component of sunlight

UV radiation that reaches the biosphere can cause damage to many biomolecules including nucleic acids, proteins and lipids. The first step in a photochemical reaction is the absorption of a single photon by a molecule (chromophore) and the production of an excited state in which one electron of the absorbing molecule is raised to a higher energy level.

The primary products generated by UV absorption are generally either reactive oxygen species in a metastable excited state or free radicals, both of which are formed fast. Dark chemical reactions occur then within μsecs, but may last for hours. Finally, these reactions are translated into photobiological responses, which may also occur rapidly but can also take years or decades to be manifested. Photons may interact directly with important biological molecules, events which mostly occur with wavelengths that fall in the UVB range or, alternatively, the energy of a photon might be absorbed by another molecule, the photosensitizer. The excited photosensitizer can then transfer its energy by various mechanisms to the target molecule. Certain photosensitising chemicals do not require oxygen to be active, but others known as photodynamic sensitizers have an oxygen requirement. Photodynamic sensitizers can lead to type I or type II photooxidation, according to how they interact with oxygen. In type I reactions, the UV-excited sensitizer molecule combines with oxygen to form a reactive radical. In type II reactions, the energy is transferred to oxygen to produce active oxygen intermediates, which in turn can interact with cellular components.

The biological effects of solar UV light change as a function of wavelength. The solar UV spectrum that reaches the surface of the earth is divided into 2 different major regions, which differ quite sharply in their biological effects- the UVB (290-320nm) and the UVA (320-380nm) regions, also known as mid- and near ultraviolet ranges. The UVB region has been thought to be the most deleterious portion of the solar UV spectrum, as it has been shown to be responsible for many damaging effects including sunburn, mutation induction, cell mortality and skin cancer. However, wavelengths in the UVA region are also potentially carcinogenic. UVA is weakly absorbed by most biomolecules but is oxidative in nature and the cellular modifications it produces

overlap considerably with those induced by oxidative damage, whereas UVB, while it does have an oxidative component, is absorbed at biologically relevant levels by critical macromolecules, including DNA and proteins. Consequently, it is a very strong DNA damaging agent. The UVB component of sunlight is several orders of magnitude more effective per unit dose (J/m^2) than the UVA, as exemplified by the UV dose producing minimal erythema (MED) in humans (Harrison and Young, 2002; Applegate and Frenk, 1995). Because of reduced molecular absorption, tissue transmission increases with wavelength, so that UVB and to a greater extent UVA radiation, reach targets well below the surface of the skin. A small but probably relevant quantity of UVA is predicted to be absorbed by blood components (Tyrrell, 1996b). UVA radiation constitutes a very significant oxidative stress and the cellular defense pathways against UVA have more in common with antioxidant pathways than with the DNA repair pathways induced primarily by the UVB radiation (for review see Tyrrell, 1995).

1.2.1 Interaction of UV with biomolecules

At shorter wavelengths, that fall in the UVB range direct absorption by cellular macromolecules including DNA and proteins, occurs with a high probability and the biological effects of DNA photodamage and mutagenesis are dominant (Pourzand and Tyrrell, 1999; Young, 1997). At longer wavelengths, which fall in the UVA range, weak absorption by most biomolecules occurs and different chromophores absorb and generate a variety of active intermediates, including reactive oxygen species (ROS)[M K T1]. Overall, UVA radiation is oxidative in nature and UVA inactivation of mammalian cells is strongly oxygen dependent, so that ROS are certainly involved in the process. As the predominant type of damage changes with wavelength, the predominant cellular defense pathways also change. Repair of damage to DNA is important at shorter wavelengths (UVB), whereas free radical scavenging pathways and cellular antioxidant systems gain importance as the wavelength increases (UVA) (Tyrrell 1996b).

1.2.1.1 Cellular chromophores

Since the chromophore is the molecule, which initially absorbs the incident UV energy, the critical chromophores will change as a function of wavelength. Peak absorption of DNA occurs at 260nm (UVC region) and its absorption drops sharply as wavelengths increase. In contrast, proteins absorb in all UV ranges depending on their composition. Several cellular components such as quinones, flavins, steroids and porphyrins are important UVA chromophores.

Protoporphyrin (PP) IX the immediate precursor to heme is a strong photosensitizer and the insertion of iron into the PPIX macrocycle produces heme, which is relatively to protoporphyrin IX, less photoactive. Iron–free porphyrins generate singlet oxygen upon irradiation. In addition, other small molecules also have the potential to generate active oxygen intermediates after absorption of UVA. An important example is the photochemical degradation of tryptophan that generates hydrogen peroxide (Tyrrell, 1991). The level of hydrogen peroxide generated *in vivo* from such a pathway would appear to be in the low micromolar range. Nevertheless, it could be crucial to biological processes, because iron complexes that are present in the cytoplasm will react with hydrogen peroxide to generate the highly reactive hydroxyl radical (in a superoxide-driven Fenton reaction). Furthermore, both hydrogen peroxide and superoxide anion are generated by UVA irradiation of NADH and NADPH. In addition, cells contain many chromophores that may generate singlet oxygen via a type II photodynamic process. In this case, the excited triplet state sensitizer will react with oxygen in the ground state to give ground state sensitizer and singlet oxygen. The most common groups of compounds of this type are the flavins, quinones and porphyrins.

1.2.2 Reactive Oxygen Species (ROS)

Molecular oxygen is an essential element for the survival of all aerobic organisms and reactive oxygen species (ROS) are generated as by-products of normal cellular metabolism, primarily in the mitochondria. The term reactive oxygen species (ROS) is a collective term that includes not only oxygen-centred radicals such as superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical (OH•), but also some nonradical derivatives of oxygen, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (for review see Halliwell and Gutteridge, 1999).

Reactive oxygen species are produced within the cell at various organelles during normal cellular metabolic processes. Any electron transferring protein or enzymatic system can result in the formation of ROS as by-products of electron transfer reactions. During aerobic energy metabolism, the oxidoreduction energy of mitochondrial electron transport chain is converted to the high-energy phosphate bond of ATP and O_2 serves as the final electron acceptor for cytochrome–*c* oxidase, the terminal enzymatic component of this mitochondrial enzymatic complex that catalyses the four electron production to H_2O. Partially reduced and highly reactive metabolites of O_2 may be produced during electron transfer reactions. These O_2 metabolites include superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), formed by one and two electron reductions of O_2, respectively. In the presence of transition metal ions the even more reactive hydroxyl radical (OH•) may be formed.

The smooth endoplasmic reticulum (ER) contains enzymes that catalyse a series of reactions to detoxify lipid–soluble drugs and other harmful metabolic products. The most extensively studied of these are the cytochrome P-450 and b_5 families of enzymes that can oxidise unsaturated fatty acids and xenobiotics and reduce molecular O_2 to produce $O_2^{\bullet-}$ and /or H_2O_2. Cytochrome oxidases and electron transport systems are also contained in nuclear membranes.

Peroxisomes are another important source of total cellular H_2O_2 production. They contain a number of H_2O_2-generating enzymes, including glycolate oxidase, D-amino acid oxidase, urate oxidase, L-α- hydroxyacid oxidase, and fatty acyl-CoA oxidase.

Only a small fraction of H_2O_2 generated in peroxisomes appears to escape peroxisomal peroxidase. In addition to intracellular membrane –associated oxidases, soluble enzymes such as xanthine oxidase, aldehyde oxidase, dihydroorotate dehydrogenase, flavoprotein dehydrogenase and tryptophan dioxygenase can generate ROS during catalytic cycling.

When cellular production of ROS overwhelms its antioxidant capacity, damage of cellular macromolecules such as lipids, proteins, and DNA may ensue. Such a state of 'oxidative stress' is thought to contribute to aging and pathogenesis of a number of diseases including cancer and those of the lung. Recent studies have implicated ROS that are generated by specialised plasma membrane oxidases in normal physiological signalling by growth factors and cytokines. The mechanism by which ROS transduce their cellular signals is not well understood. The emerging concept on the signal transduction by ROS involves alterations in the cellular redox state and the oxidative modification of proteins (for review see Thannickal and Farnburg, 2000; Dalton *et al.*, 1999).

In a variety of non-phagocytic cells types including fibroblasts, there is a marked rise of intracellular (ROS) including O_2^- and H_2O_2, after activation of Platelet derived Growth Factor (PDGF) receptor or Epidermal growth factor (EGF) receptor stimulation. This is dependent upon the small GTP-binding protein Rac1 and phosphatidylinositol 3 kinase (PI3K) (Irani *et al.*, 1997; Sundaresan, 1996; Bae, 1999; Bae, 2000). It has been shown that UVA irradiation leads to an, at least partial, functional EGF- and PDGF- receptor autophosphorylation (Schlessinger, 2000) and that this is dose dependent. It has been suggested that the mechanism for this may be related to a ROS mediated inhibition of tyrosine dephosphorylation of receptor tyrosine kinases (RTKs) by inactivation of membrane bound protein tyrosine phosphatases (Knebel, 1996). ROS synthesis is diminished in keratinocytes in which the integrity of membrane domains was disrupted by cholesterol sequestration with methyl-β-cyclodextrin and fillipin. A reduced level of ROS synthesis was also shown in UVA irradiated cells treated with these agents relative to controls (Gniadecki *et al.*, 2002)

1.2.2.1 Singlet Oxygen ($^1O_2$)

Singlet molecular oxygen ($^1O_2$) is a highly reactive form of molecular oxygen that may oxidise critical organic molecules (Vile and Tyrrell, 1995). $^1O_2$ is a derivative of molecular oxygen in which all valence electrons are spin paired. The electronic configuration of singlet oxygen ($^1\Delta g$, $^1O_2$) is simplified as $(\pi^*2p_x)^2$, $(\pi^*2p_y)^0$, illustrating its non radical nature. Singlet oxygen exists in two forms ($^1\Delta g$, $^1O_2$) and a higher energy form (Σg^+, $^1O_2$), which decays to the $^1\Delta g$, $^1O_2$ immediately upon formation and is thus thought to irrelevant to biological systems.

The lifetime of $^1O_2$ is approximately 10 fold longer in deuterium oxide (D_2O) than in water or aqueous solutions (Merkel and Kearns, 1972). Thus, biological responses are often compared by replacing H_2O in a system with D_2O, as a test for $^1O_2$ involvement. Enhancement of a biological reaction in this way may be taken as strong evidence of $^1O_2$ involvement, assuming that isotope effects are negligible (Ryter and Tyrrell, 1998; Pourzand *et al.*, 2000). Other tests for detecting $^1O_2$ involvement in reactions is the use of agents that function as $^1O_2$ quenchers of varying specificities such as 1,4-diazabicyclooctane (DABCO), β-carotene, sodium azide (NaN_3) and L-histidine or chemical traps such as 1,3-diphenylisobenzofuran and cholesterol (Kulig and Smith, 1973; Oosthuizen and Greyling, 1999; Osada and Sevanian, 2000). The addition of these compounds should inhibit a reaction dependent on $^1O_2$. If these compounds do not inhibit the biological effect under study when added at adequate concentrations, it is likely that $^1O_2$ is not involved in the process. However, if the given effect is quenched by these scavengers in cellular systems, this does not provide absolute proof of $^1O_2$ involvement, as these compounds are not totally specific for $^1O_2$ and they could react with other reactive oxygen species (ROS) or strong oxidants and $^1O_2$ could be quenched by competing organic molecules (Ryter and Tyrrell, 1998).

Overall, several parameters have to be taken into account. For example, azide, although a $^1O_2$ scavenger has also many other cellular effects including inhibition of RNA polymerase (Tyrrell, 1997) so this must be considered if the biological end-point is dependent on transcription. The quenching by L-histidine also provides valuable information about the superoxide anion involvement, since it has a negligible

interaction with superoxide anion and quenching by this compound indicates that the involvement of this anion is unlikely. Both azide and histidine react with very reactive radicals (such as hydroxyl radical) so that quenching alone is not diagnostic. Furthermore, DABCO and diphenylisobenzofuran also react with $RO_2^{\cdot}$ radicals (for review see Pourzand *et al.*, 2000). As corroborative evidence for $^1O_2$ involvement in a given effect, the effect should be also seen after appropriate generation of this intermediate. It is has been proposed that the best way to generate this compound is to irradiate a sensitizer (e.g. Rose Bengal) attached to beads or to a membrane in close vicinity to the target cells but with an air interface (for review see Tyrrell, 1997; Ryter and Tyrrell, 1998).

1.2.2.2 UVA effects mediated by singlet oxygen

Many of the damaging effects of UVA radiation in vitro can be attributed to the generation of singlet oxygen (Tyrrell, 2000). This intermediate is clearly a major factor in mediating UVA peroxidation of membrane lipids (Vile and Tyrrell, 1995), in UVA mediated DNA damage (Peak *et al.*, 1987; Kvam and Tyrrell, 1997) and in UVA induced necrotic cell death (Tyrrell and Pidoux, 1989). There is also evidence that this agent is involved in UVA activation of gene expression. Such evidence exists for the UVA dependent activation of heme oxygenase (Basu Modak and Tyrrell, 1993). Similar data exist for activation of the collagenase gene (MMP-1) in human fibroblasts (Wlaschek *et al.*, 1997). Singlet oxygen has also been implicated in UVA mediated activation of signal transduction pathways, including p38 and c-jun-N-terminal kinases but not extracellular regulated kinases (Klotz *et al.*, 1999; 1997).

1.2.3 Protein damage by UVA

Studies at the beginning of the 20th century showed that certain enzymes, including amylase, catalase, pepsin and tyrosinase are inactivated in an oxygen-dependent process by UVA irradiation (Agulhon, 1912). Proteins containing cystine residues are the most

labile targets because of the high reactivity of the sulphide bond but aromatic amino acids (tryptophan, phenylalanine, and tyrosine) are also sensitive because they absorb in the UV range (see Tyrrell 1984). Among the antioxidant enzymes, both peroxidase and catalase are heme containing proteins which are UVA chromophores and may generate active oxygen species. Catalase exhibits an absorption maximum in the UVA range with a peak close to 400nm. Studies have shown that it is rapidly destroyed by UVA radiation (Mitchell and Andersen, 1965) and that it may act as an endogenous photosensitizer (Eisenstark and Perrot, 1987).

Oxygen radicals and other activated oxygen species cause modifications of the amino acids of proteins that frequently result in functional changes of structural or enzymatic proteins (Stadtman, 1992). Protein carbonyls may be formed either by oxidative cleavage of proteins or by direct oxidation of lysine, arginine, proline, and threonine residues (Levine *et al.*, 1994). Oxidative modification of proteins appears to mark them for degradation (Stadtman, 1990).

1.2.4 DNA damage

Wavelengths in the UVA waveband cause various types of DNA damage, including cyclobutane-type pyrimidine dimers (Tyrrell, 1973), strand breaks (Peak *et al.*, 1987; Rosenstein, 1983) and DNA protein crosslinks (Peak and Peak, 1991). The latter two classes become more important as wavelength increases. Dimer formation does not appear to be influenced by reducing the oxygen tension, so active oxygen species are unlikely to be involved. However, fluence dependent induction of DNA strand breaks in the DNA of *Escherichia coli* is completely prevented by irradiation under anaerobic conditions (Tyrrell *et al.*, 1974), although this damage does not appear to contribute to cell death. It has also been shown that strand break induction by irradiation (365nm) of human cells is enhanced in the presence of deuterium oxide, an agent that increases the singlet oxygen lifetime, possibly implicating singlet oxygen (Peak *et al.*, 1987).

1.2.5 UVA mediated membrane damage

The lipid membrane is readily susceptible to attack by active oxygen intermediates. Lipid peroxidation and peroxidative breakdown of membranes are well established as types of damage induced by UVA (Niki *et al.*, 1991; Skoog *et al.*, 1997) and these effects are known to be catalysed by heme proteins such as cytochrome c and catalase (Tyrrell, 1991; Applegate and Frenk, 1995). Furthermore, UV induces changes in the membrane's fluidity (Tyrrell, 1991; Gaboriau, 1993). An agent that enhances singlet oxygen lifetime, D_2O, enhances the level of membrane damage, sensitivity to UVA and lipid peroxidation (Chamberlain and Moss, 1987; Vile and Tyrrell, 1995).

Peroxidation of membrane lipids can occur nonenzymatically as well as enzymatically in UVA irradiated mammalian cells (Vile and Tyrrell, 1995; Hanson and DeLeo, 1990). During non- enzymatic peroxidation, active oxygen species initiate the process and the resulting lipid radicals propagate the peroxidation process, which lead to an accumulation of lipid hydroperoxides. Their decomposition leads to a variety of end products, the major being malonaldehyde (MDA), hexanal and 4-hydroxynonenal (4-HNE). 4-HNE activates adenylate cyclase and phosphatidyl inositol 4,5 biphosphate phospholipase C ($PI(4,5)P_2$-phospholipase C) (Rossi *et al.*, 2001) and phospholipase D (Natarajan *et al.*, 1993) in cells. Adenylate cyclase increases the intracellular concentration of cAMP, which activates cAMP dependent kinases. $PI(4,5)P_2$-phospholipase C catalyses the hydrolysis of PIP_2 to IP_3 and diacylglycerol (DAG). In enzymatic lipid peroxidation oxidised fatty acids are released from membrane lipids by phospholipases and other lipases. UVA irradiation stimulates arachidonic acid (AA) release from membrane phospholipids. Arachidonic acid, which is released by phospholipase A_2, is a substrate for synthesis of eicosanoids by lipoxygenases (LOXs), cycloxygenases (COXs) and epoxigenases, namely leukotrienes, prostaglandins and thromboxane. The cyclooxygenase metabolites of arachidonic acid could then activate signal transduction events involved in UVA mediated HO-1 induction (Basu-Modak *et al.*, 1996).

It has been shown that a variety of proteins involved in cell signaling are concentrated in cholesterol-rich domains of the membrane designated "membrane rafts" or "lipid

rafts" (Simons and Ikonen, 1997; Simons and Toomre, 2000). It is thought that "membrane rafts" function as sites of assembly of proteins involved in cell signaling including cell surface receptors, GPI- anchored proteins, Src kinases, and Ras proteins (Brown and London, 2000). One subset of lipid rafts is found in cell surface invaginations called caveolae. Caveolae are formed from lipid rafts by polymerisation of caveolins- integral membrane proteins that tightly bind cholesterol. Cholesterol is a major constituent of lipid rafts and caveolae, which are sites where signalling molecules are concentrated. Recently a model has been suggested to describe the physical properties of cholesterol/phospholipid/glycophospholipid mixtures that must play an important role in the regulation of their synthesis, transport and localisation in cell membranes (Radhakrishnan *et al.*, 2000).

In monolayers, there is experimental and theoretical evidence for a strong (step-like) increase of cholesterol chemical activity on increasing cholesterol concentration beyond the complex stoichiometric composition. It is thought that the "condensed complexes rich phase" is likely to be present in lipid rafts of animal cell membranes and has a major effect in determining the chemical activity of cholesterol, which affects its binding to proteins. A putative raft domain may include many hundreds of complexes along with small amounts of uncomplexed lipids (Radhakrishnan *et al.*, 2000). It is suggested that the chemical activity of cholesterol plays an essential role in the regulation of cholesterol biosynthesis (Radhakrishnan and McConnell, 2000).

To perform these functions, membrane cholesterol must be kept at constant level (Brown and Goldstein, 1999). This homeostasis is achieved by a feedback regulatory system that senses the level of cholesterol in cell membranes and modulates the transcription of genes encoding enzymes of cholesterol, unsaturated fatty acid and triglyceride biosynthesis and uptake from plasma lipoproteins. The modulators are a family of endoplasmic reticulum membrane-bound transcription factors, called sterol regulatory element binding proteins (SREBPs) that activate genes encoding enzymes of cholesterol and fatty acid biosynthesis in sterol depleted cells. This activation of transcription is blocked when sterols build up in cells. SREBPs need to be released proteolytically from membranes to act. Following cleavage, the NH2 –terminal domain of SREBPs is released and travels to the nucleus, where it binds to SREs in the

enhancers of target genes. The NH2 –terminal domains of SREBPs are transcription factors of the basic-loop-helix-leucine zipper (bHLH-Zip) family. The extreme NH2 – terminus contains a stretch of acidic amino acids that recruits coactivators including CBP (Brown and Goldstein, 1999; Swinnen *et al.*, 1997; Scheek *et al.*, 1997; Edwards *et al.*, 2000).

The most important role of lipid rafts at the cell surface may be their function in signal transduction. Lipid rafts containing a given set of proteins can change their size and composition in response to stimuli. This favours specific protein–protein interactions, resulting in the activation of signalling cascades. As an example, in the case of tyrosine kinase signalling (e.g. EGFR activation by UVA), adaptors, scaffolds and enzymes are recruited to the cytoplasmic side of the plasma membrane as a result of activation. Even a small change of partitioning into rafts can initiate signaling cascades (Simons and Ikonen, 2000).

One way to consider rafts is that they form concentrating platforms for individual receptors activated from ligand binding. In general, raft binding recruits proteins to a new micro-environment, where the phosphorylation state can be modified by local kinases and phosphatases resulting in downstream signalling (Simons and Ikonen, 1997; Simons and Toomre, 2000).

1.2.6 Inactivation of mammalian cells

Populations of human cells (Hela) or Chinese hamster cells are inactivated in a salts buffer by monochromatic radiation in the UVA range (365nm) (Danpure and Tyrrell, 1976). The inactivation is strongly dependent upon the presence of oxygen, suggesting that active oxygen intermediates are involved in the observed effect. Furthermore, irradiation in the presence of sodium azide (NaN_3 - a quencher of singlet oxygen) or D_2O (which enhances the singlet oxygen lifetime) lead to significant levels of protection or sensitisation, respectively.

1.2.7 UVA irradiation and constitutive cellular defense pathways

1.2.7.1 Small antioxidant molecules

Glutathione (GSH) is present in most mammalian cells at high concentrations and is believed to play a crucial role in protection of cells against oxidative damage. GSH is synthesized from its constituent amino acids by a sequential action of γ-glutamylcysteine synthetase (γ-CGS) and GSH synthetase. The activity of γ-CGS determines the rate of GSH synthesis and is feedback inhibited by GSH (Meister and Anderson, 1983; Rahman and MacNee, 2000). One of the immediate cellular effects of physiologically relevant UVA doses is depletion of cellular GSH content (Wheeler *et al.* 1986; Connor and Wheeler, 1987) and this is not affected by the presence of lipophilic antioxidants (Basu-Modak *et al.*, 1996; Ossola and Tomaro, 1998). In cell culture models using human cells it has been shown that glutathione depletion, using D,L-buthionine-*S,R*-sulfoxime (BSO) (an inhibitor of γ-CGS), sensitizes cells to UVA (Tyrrell and Pidoux, 1986; 1988). Glutathione provides an important intracellular free radical scavenging system and constitutes the most abundant non-protein free thiol in mammalian cells. Additionally GSH is also a cofactor for antioxidant enzymes important in drug detoxification such as glutathione peroxidase, transhydrogenases and glutathione transferases (Applegate and Frenk, 1995). The protection it affords to UVA challenged cells, might be due to its action as a unique hydrogen donor for glutathione peroxidase (Tyrrell and Pidoux, 1988).

Recently, a different and new physiological role has been defined for these compounds because of their involvement in nitric oxide binding and transport in biological systems (Foresti *et al.*, 1997). It appears that NO is stabilised by thiols through the formation of S-nitrosothiols and a dynamic equilibrium among free NO, O_2^- and endogenous glutathione might constitute an interactive signalling mechanism modulating stress and adaptive responses in tissues.

Thiols also play an important role by interacting with the oxidised cysteine residues in the active center of protein tyrosine phosphatases (PTPs) and reversing their

inactivation by UVA and metal toxins. UVA interacts with receptor tyrosine kinases (RTKs) (e.g. Epidermal growth factor (EGFR) and platelet-derived growth factor receptor (PDGFR) (Knebel *et al*, 1996) that initiate intracellular phoshorylating cascades counteracted by action of regulatory molecules that involve protein tyrosine phosphatases (PTPs). The "repair" of PTPs by thiols is of physiological significance (Aslund and Beckwith, 1999; Herrlich and Boehmer, 2000).

1.2.7.2 Antioxidant enzymes

The major classes of antioxidant enzymes include superoxide dismutases (SODs), catalase (CAT) and glutathione peroxidases (GSH-Px).

Superoxide dismutase enzymes (SODs) remove $O2^{\bullet-}$ by accelerating its conversion to H_2O_2. Human cells have a SOD enzyme containing manganese at its active site (MnSOD) in the mitochondria. A SOD with copper and zinc at the active site (CuZnSOD) is also present, but largely in the cytosol. Catalase enzymes convert H_2O_2 to water and O_2. However, it is noteworthy that neither SODs nor CAT alone play major role in protecting cells from the lethal effects of UVA irradiation (Tyrrell and Pidoux, 1989).

On the other hand, glutathione peroxidases (GSH-Px) (selenium glutathione peroxidase and phospholipid hydroperoxide peroxidase) are important enzymes in the cellular defense against UVA induced damage (Tyrrell and Pidoux, 1988; Lautier *et al.*, 1992; Jornot and Junot, 1993). Glutathione peroxidase (GPx) catalyzes the conversion of UV-induced H_2O_2 in to water and oxygen and the reduction of toxic lipid peroxides to less toxic hydroxy fatty acids utilizing glutathione as a co-factor. Selenium-dependent GPx exhibits high reactivity toward both H_2O_2 and organic hydroperoxides and it is found both in cytosol and mitochondria. The activity of this enzyme is not strongly affected by UV and is considered to be a most important antioxidant defense system in the skin (Shindo *et al.*, 1994; Halliwell and Gutteridge, 1999).

Whereas high UV doses and ROS levels are known to overwhelm the antioxidant enzymatic defense system, clinical studies have shown that repetitive low UV doses prevent phototoxicity and UV-induced pathologies upon subsequent higher UV doses, (Rucker *et al.*, 1991) indirectly suggesting an inducible adaptive antioxidant defense response.

1.2.8 UVA induced gene expression

UVA is effective at inducing expression of numerous genes in human cells. These genes include collagenase (Scharffetter-Kochanek *et al.*, 1993); IL-1 and IL-6 (Wlaschek *et al.*, 1997), ICAM-1 (Krutmann *et al.*, 1995), c-fos and c-jun (Soriani *et al.*, 2000), heme oxygenase–1, cyclooxygenase 2 (for review see Tyrrell, 1994; 1996; 1996b) inducible nitric oxide synthase (Suschek *et al.*, 2001) and manganese superoxide dismutase (MnSOD) (Possig *et al.*, 1999).

Another UVA induced gene is CL-100, whose transcription is also strongly activated by oxidants (Keyse, 1995). CL-100 encodes a dual specificity (tyrosine/threonine) protein phosphatase that specifically inactivates cellular mitogen –activated protein (MAP) kinases and is therefore implicated in the modulation of signal transduction events involved in the cellular stress response (Keyse, 1995; Tyrrell, 1996b).

Also the inducible form of cyclooxygenase, cyclooxygenase 2, has been shown to be induced in normal human skin fibroblasts by UVA (Feng *et al.*, 1995; Soriani *et al.*, 1998).

UVA has been shown to activate activator protein 1 (AP-1) (c-Fos/c-Jun) in a human keratinocyte line (Silvers *et al.*, 2002) and in fibroblasts (Nakano *et al.*, 2001). AP-1 activity is known to play a key role in the regulated expression of important components of the cytokine network, cell surface receptors and proteases, which orchestrate the process of wound healing (for review see Angel *et al.*, 2001; Sen and Packer, 1996; Allen and Tresini, 2000).

1.3 Heme oxygenase –1

Heme oxygenase (HO) is the major heme-degrading enzyme and provides a vital physiologic function in regulating the intracellular concentration of heme in mammalian tissues. HO catalyses the first and rate limiting step in the degradation of heme in eukaryotic cells, leading to the formation of biliverdin IXα, carbon monoxide (CO), and free iron. Biliverdin is subsequently converted to bilirubin with action of biliverdin reductase and free iron is sequestered in ferritin. The degradation of heme by HO requires the action of the microsomal enzyme NADPH cytochrome cP450 reductase, which serves to donate electrons to the reaction. The demonstration that oxidative stress in general was causing strong transcriptional activation of the gene led to the hypothesis of a new function of HO-1 in cellular defense. The reaction products of HO activity, biliverdin and its subsequent metabolite bilirubin, have antioxidant properties. Potential pro-oxidant consequences of HO activity are related to the production of iron, which could be involved in reactions that compete with iron reutilization and sequestration pathways (for review see Ryter and Tyrrell, 2000).

Three mammalian isoforms of heme oxygenase (HO) that catalyse the degradation of heme have been identified to date: heme oxygenase-1 (HO-1), heme oxygenase-2 (HO-2) and heme oxygenase-3 (HO-3) (McCourbey *et al.*, 1997; Otterbein *et al.*, 2000). They are products of three different genes. HO-1 is a 32-kDa protein that is induced in response to numerous stimuli.

Although heme is the typical HO-1 inducer it has been clearly shown that HO-1 activity can also be stimulated by a variety of non-heme inducers including UVA irradiation, endotoxin, heavy metals and oxidants, such as hydrogen peroxide (Keyse and Tyrrell, 1989; Keyse *et al.*, 1990; Vile and Tyrrell, 1993; Ossola and Tomaro, 1998). Glutathione depletion strongly enhances both, basal and oxidant induced expression of HO-1 (Lautier *et al.*, 1992; Pourzand *et al.*, 2000; Rahman and MacNee, 2000). The enhanced expression of the HO-1 gene by UVA and other agents can be accounted for by a strong enhancement in transcription rate (Keyse *et al.*, 1990). Redox signaling appears to play a major role in HO-1 regulation, however various redox-independent pathways are also involved in HO-1 gene regulation (Maines, 1996).

This enzyme is most highly concentrated in tissues that are heavily involved in the catabolism of heme proteins. HO-2, a 36-kDa protein is a non-inducible isoform that is present in highest concentrations in brain and testes. HO-3, a 33-kDa protein, also catalyses heme degradation, but with a much lower catalytic activity than the HO-2 protein isoform. The physiologic role of this protein remains uncertain (Galbraith, 1999; Ortiz de Montelano, 2000). The three isoforms have little in common in primary structure, regulation, or tissue distribution. In mammals the measured heme oxygenase activity is the sum of HO-1, HO-2 and HO-3 activities (Maines, 1997).

The carbon monoxide (CO) released from heme during HO activity may function as a soluble second messenger gas molecule in a fashion that resembles that of the free radical gas nitric oxide ($NO^{\bullet}$) (Verma *et al.*, 1993). In contrast to NO, however, which forms peroxynitrite with superoxide, CO does not form radicals. In the liver, CO is involved in the regulation of cytochrome P450-dependent biotransformation. HO-1-derived CO has been shown to protect the hepatic microcirculation under stress conditions (Suematsu *et al.*, 2000). Furthermore, CO appears to play a prominent regulatory role for the tone of the cardiovascular system by promoting vasodilatation (Sammut *et al.*, 1998)

It has been shown that up-regulation of transcription of the HO-1 gene by heme was reduced after an initial exposure to heme. In this study, a second exposure to heme: albumin given at a time when HO-1 mRNA accumulation was highest following the first induction led to a lower than expected transcriptional activation. UVA also leads to development of refractoriness to activation of this gene (Noel and Tyrrell, 1997).

The immediate release of heme, the heme oxygenase substrate, from microsomal proteins by UVA irradiation of human skin fibroblasts, has been suggested to occur via a cyclooxygenase dependent mechanism, since indomethacin an inhibitor of cyclooxygenase 1 and 2 (Willoughby *et al*, 2000; Mitchell and Warner, 1999) inhibited the UVA dependent release of heme (Kvam *et al.*, 1999). HO not only controls the availability of heme for the synthesis of heme proteins, but also is responsible for the generation of CO, which binds to the heme moiety of heme proteins thus affecting their enzymatic activity. It has been shown (Haider *et al.*, 2002) that the heme-HO system

regulates COX enzyme expression and activity in rabbit vascular endothelial cells{PRIVATE "TYPE=PICT;ALT=alpha "} and that the increase in HO activity was associated with a subsequent decrease in COX activity, which returned to normal levels following normalisation of HO activity. {PRIVATE "TYPE=PICT;ALT=alpha "} The degree of HO-1 expression and, consequently, the level of cellular heme, were directly related to COX activity. The authors concluded that the heme-HO system can function as a cellular regulator of the expression of vascular COX, thus influencing the generation of prostanoids, PGE_2 and PGI_2, known to play a role in vascular homeostasis. A similar finding was published regarding the regulation of COX activity by the HO system in rats (Botros *et al.*, 2002).

HO-1 induction has also been implicated in a photoimmunoprotective activity of UVA in the skin of UVB irradiated mice (Reeve and Tyrrell, 1999). Furthermore, HO-1 is induced in a number of experimental injuries and diseases including congestive heart failure (Raju *et al.*, 1999), kidney reperfusion injury (Raju and Maines, 1996), corneal inflammation (Laniado *et al.*, 1997) and ischemic stroke (Panahian *et al.*, 1999).

There is strong evidence that the induction of HO-1 has therapeutic implications in diseases associated with oxidative stress (for review see Immenschuh and Ramadori, 2000). However, it has also been shown that HO-1 gene activation is not protective under certain experimental circumstances (Platt and Nath, 1998; Suttner and Dennery, 1999; Lamb *et al.*, 1999; Nutter *et al.*, 1994; Dennery *et al.*, 2003). The therapeutic potential of targeted HO-1 gene transfer has been investigated in diseases such as ischemic stroke (Panahian *et al.*, 1999) and inflammatory lung injury caused by hyperoxia (Otterbein *et al.*, 1999). A significant therapeutic potential of modulation of HO-1 gene induction has been implicated in various immune functions, particularly important in transplantation medicine (DeBruyne *et al.*, 2000).

1.4 Carotenoids

Carotenoids are a class of hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls) with more than 600 naturally occurring members known to date. They are important plant pigments found in the photosynthetic pigment protein complex of plants, photosynthetic bacteria and algae and are responsible for the bright colours of many fruits, vegetables and flowers, as well as for the colours of many birds and marine animals. Carotenoids are unique in that their diversity of function is unmatched by any other class of pigments found in photosynthetic organisms (Faure *et al.*, 1999).

The carotenoids are not synthesised in animals and their circulating levels in humans are a result of dietary intake (Rock, 1997; IARC Working Group for the evaluation for cancer, 1998; Faure *et al.*, 1999; Stahl and Sies, 1996). Approximately 60 carotenoids can be metabolised to retinol and function as Vitamin A precursors in mammals. The predominant carotenoids observed in the plasma are β-carotene, lycopene, lutein, β-cryptoxanthin and α-carotene, which account for 90% or more of the circulating carotenoids in humans (Rock, 1997).

Carotenoids consist of eight isoprenoid units joined in such a manner that the arrangement is reversed at the centre of the molecule so that the two central methyl groups are in a 1,6-positional relationship and that the remaining non-terminal methyl groups are in a 1,5-positional relationship. The most striking and characteristic feature of the carotenoid structure is the long system of alternating double and single bonds that they carry (Britton, 1995). This constitutes a conjugated system in which the π-electrons are effectively delocalised over the entire length of the polyene chain. All carotenoids may be formally derived from the acyclic $C_{40}H_{56}$ structure (lycopene) illustrated in **Fig. 1.4-1.** after steps of hydrogenation, dehydrogenation, cyclisation, oxidation or any combination of these processes. The International Union of Pure and Applied Chemistry (IUPAC) and the IUPAC-International Union of Biochemistry Commissions on Biochemical nomenclature have published rules for the nomenclature of carotenoids. All specific names are based on the stem name "carotene", which corresponds to the structure and numbering shown in **Fig. 1.4-2.** The name of a specific compound is constructed by adding as prefixes two Greek letters that specify the two end C_9 end

groups **Fig. 1.4-3.** The structures of some important retinoids can be seen in **Fig. 1.4-4**. Chirality and geometric configuration are designated as *R/S* or *E/Z* respectively (IARC Working Group for the Evaluation of Cancer, 1998). In this thesis, the term trans is also used for the E and cis for the Z geometric configuration. Derivatives in which the carbon skeleton has been shortened by the formal removal of fragments from one end of a carotenoid are named "apocarotenoids" the position of the point of cleavage being indicated e.g. β-apo-8'-apocarotenal.

The cis-trans isomerism of the carbon–carbon double bonds is an important feature, because these geometric isomers may have different biological properties. These forms may be interconverted by light, thermal energy, or chemical reactions (Rock, 1997). Carotenoids usually occur in nature as all-trans isomers and many cis isomers that are described in the literature, as natural products may be artefacts (IARC Working Group for the Evaluation of Cancer, 1998).

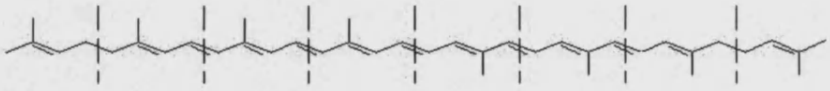


Fig. 1.4-1. The basic carotenoid skeleton, constructed from eight isoprenoid units (The figure is reproduced from IARC Working Group for the Evaluation of Cancer, 1998)

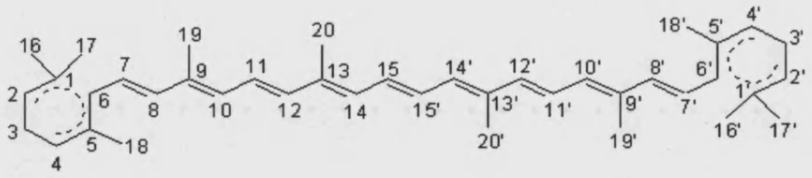


Fig. 1.4-2. Numbering scheme for carotenoids (The figure is reproduced from IARC Working Group for the Evaluation of Cancer, 1998)

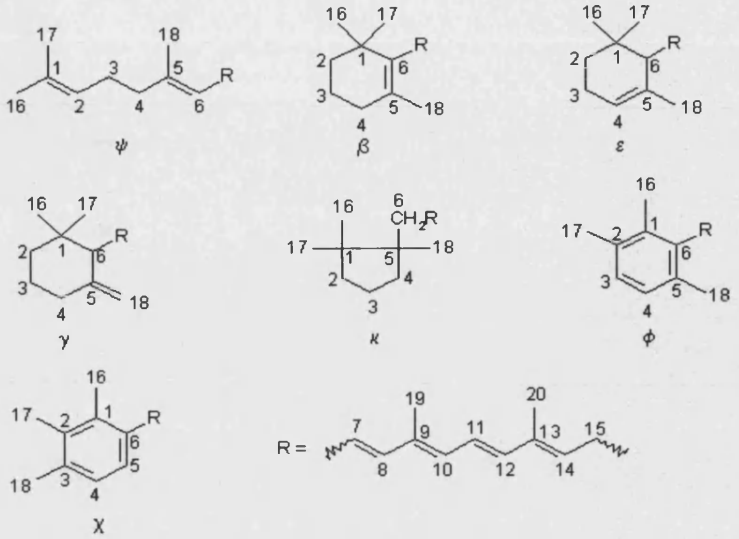


Fig. 1.4-3. The seven end groups in carotenoids (The figure is reproduced from IARC working Group for the Evaluation of Cancer, 1998)

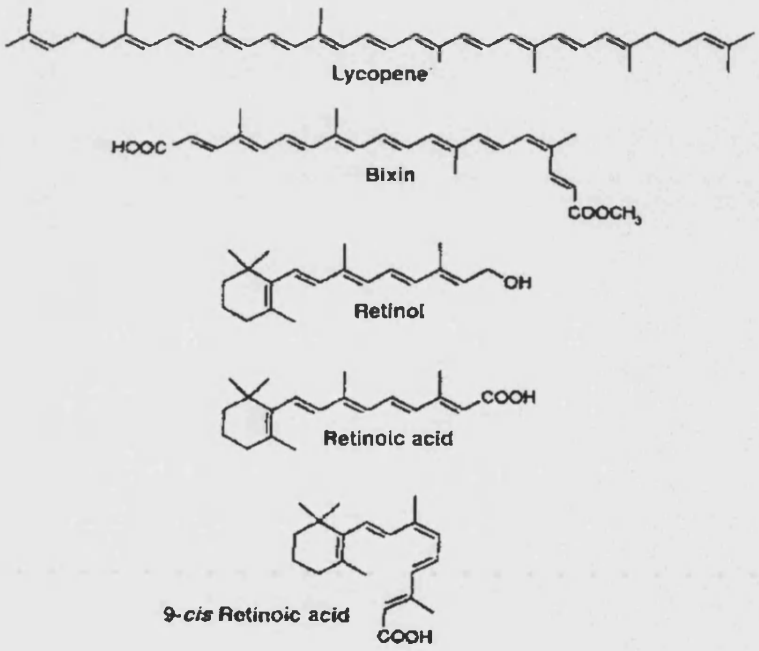


Fig. 1.4-4. Structures of important retinoids (The figure is reproduced from IARC working Group for the evaluation of Cancer, 1998)

1.4.1 General properties of carotenes

The natural functions and biological actions of carotenoids are determined by the physical and chemical properties of the molecules, which are defined by their molecular structure. All carotenoids in the all-trans configuration are linear, rigid molecules. The conjugated double bond system also determines their chemical and photochemical properties that form the basis of their biological properties. A third factor is interaction between carotenoids and other molecules in their immediate vicinity, which can greatly alter the properties of the carotenoids and thus affect their functioning in vivo. The carotenes are a group of extremely hydrophobic molecules with no solubility in water. They are thus expected to be restricted to hydrophobic areas in the cell, such as the inner core of membranes, except when association with proteins allows them to access an aqueous environment (Gruszecki, 1999). Although their shape in the all-trans configuration is linear, the overall shape of the cis isomers differs substantially from that of the all-trans form, so their ability to fit in subcellular structures may differ. The shape and size of end groups are also important factors. Acyclic carotenoids such as lycopene are essentially long, linear molecules with flexible end groups. Cyclisation shortens the overall length of the molecule and the space they occupy (Britton, 1995).

1.4.2 Light absorption

The absorption of light energy by an organic molecule produces a higher-energy excited state of that molecule. In the case of carotenoids the relevant transition is a $\pi \rightarrow \pi^*$ transition, in which on of the bonding π-electrons of the conjugated double bonds is promoted to a previously unoccupied π^* antibonding orbital. The π-electrons are highly delocalised and the excited state is of comparatively low energy. The energy needed for the transition is small and corresponds to light in the visible region in the wavelength range of 400-500nm. Carotenoids are therefore intensely coloured yellow, orange or red. The relationship between chromophore and light absorption properties is used for their identification (Britton, 1995). The characteristic strong absorbance of light in the *vis* is attributed to a transition from the ground state S_0 to the second singlet excited

state S_2 (1^1Bu). The energy levels of the S_2 state and the somewhat lower first excited state S_1 (1^1Ag) that can be formed from its internal conversion, lie close to but above those of chlorophyll, so that singlet-singlet energy transfer can take place form excited carotenoid to generate the excited singlet state chlorophyll S_1, which is active in photosynthesis. Direct formation of the carotenoid triplet state (T_1) from the excited singlet states S_2, or S_1 via singlet –triplet intersystem crossing is not favoured. Its formation by energy transfer from other triplet state molecules e.g. photosensitizers can be very efficient, provided that that the carotenoid contains more than seven conjugated double bonds, because the carotenoid triplet-state energy is low. Transfer of energy from triplet state chlorophyll and other porphyrins to carotenoids occurs much more readily than the alternative energy transfer to oxygen to form singlet oxygen. Carotenoids can also accept excitation energy from $^1O_2$. The triplet state carotenoid is of such low energy that it is unable to form other species by energy transfer and dissipates its excitation energy to its surroundings (for review see Britton, 1995).

1.4.3 Chemical properties

The conjugated polyene system is a reactive electron-rich system that is susceptible to attack by electrophilic reagents and is responsible for the instability of carotenoids towards oxidation. It is the most important part of the molecule in relation to free-radical chemistry (Britton, 1995). Pure carotenoids, even in the crystalline state, are susceptible after isolation to oxidation and may be broken down rapidly if samples are stored in the presence of even traces of oxygen. In vivo, however, the carotenoids are usually stabilised to a considerable degree by proteins and other molecules in their immediate vicinity. Even in vivo carotenoids are still susceptible to oxidative damage if they become exposed to oxidising species. Oxidative breakdown is also caused by free radical reactions; rapid bleaching by hydroxyl radicals is well known (Young and Lowe, 2001; Krinsky, 1989).

Recent work has concentrated on the reactions of carotenoids with peroxy radicals generated in some cases by pulse radiolysis mostly by use of azo-initiators such as 2,2'-azo-bis-2-4-dimethyl valeronitrile (AMVN) (Woodall *et al.,* 1997, Young and Lowe, 2001). A wide variety of products have been detected in these studies. Most seem to be

apocarotenals or apocarotenones of various chain lengths produced by cleavage of any double bond in the polyene chain; epoxides are also commonly found (IARC Working Group for the valuation of Cancer, 1998).

Similar chemical reactions occur between carotenoids and singlet oxygen and again produce apocarotenals and apocarotenones as the major products. The mechanism though might be different and may involve the addition of $^1O_2$ to a double bond to form a labile dioxetane, which is then cleaved to give the corresponding carbonyl products.

Carotenoids are rapidly destroyed by oxidation, especially by free radicals such as the hydroxy radical and various peroxy radicals. The important fundamental chemistry of carotenoid radicals and of the reaction of carotenoids with oxidising free radicals is not well understood. Carotenoid radicals are charged or neutral and are very short lived species. In principle, removal of one electron from the carotenoid molecule by oxidation gives the radical cation $Car^{+\bullet}$ whereas the addition of one electron by reduction generates the radical anion $Car^{-\bullet}$. These charged radicals can be detected by their distinctive spectroscopic properties in the near infrared region. The abstraction of a hydrogen atom from a saturated carbon atom in a position allylic to the polyene chain, by homolytic cleavage, would generate a neutral radical $Car^{\bullet}$. In all these radicals, delocalisation of the unpaired electron over the conjugated polyene chromophore has a stabilising effect and allows subsequent reactions to take place in many parts of the molecule. The same is true for carotenoid –adduct radicals, which could be generated by the addition of a radical species such as a peroxy radical $ROO^{\bullet}$ or a hydroxy radical $HO^{\bullet}$ to the polyene chain. A series of apocarotenoids of different chain length have been reported to be products of chemical and free radical oxidation of carotenoids. They could be produced in tissues by non-specific chemical or enzymatic reactions or by specific excentric cleavage of carotenoids in the intestine (for review see IARC Working Group for the Evaluation of Cancer, 1998).

Burton and Ingold (1984) have shown that the radical trapping antioxidant behaviour of β-carotene in chlorobenzene changes as a function of oxygen partial pressure so that at high oxygen pressure it loses its antioxidant activity especially at high concentrations.

This is especially important regarding the effects of carotenoids in humans since low partial pressures are found in most tissues under physiological conditions.

1.4.4 Singlet Oxygen Quenching by Carotenoids

An important feature of carotenoids is their ability to quench singlet oxygen. Numerous in vitro studies have been employed to unravel the mechanism by which β-carotene and other carotenoids quench singlet oxygen.

Two major mechanisms have been proposed in the literature, physical and chemical quenching, and it is likely that both contribute to an extent. Most commonly, carotenoids catalytically quench singlet oxygen through an almost diffusion-controlled energy transfer process (physical pathway). Physical quenching involves the transfer of excitation energy from $^1O_2$ to the carotenoid thereby producing ground state oxygen and a carotenoid in the excited triplet state. The excess energy is dissipated as heat through rotational and vibrational interactions with the surrounding solvent and structures. The regeneration of the ground state carotenoid allows it to function as a catalyst and undergo additional cycles of $^1O_2$ quenching. In contrast to physical quenching, less efficient but concomitant processes exist involving real chemical reactions (Garavelli *et al.*, 1998).

Quenching constants for $^1O_2$ have been determined for a range of carotenoids in solution and a relationship between the length of the conjugated C=C chain and ability to quench $^1O_2$ by energy transfer has been demonstrated. This ability is also influenced by the end- (e.g. for β-carotene and lycopene) and substituent groups (DiMascio *et al.*, 1989; Conn *et al.*, 1991; Stahl and Sies, 1993). The singlet oxygen quenching second order rate constants for different carotenoids in benzene can be seen in **Table 1.4.4-1** (Edge and Truscott, 1999). Carotenoids may undergo trans- or cis-isomer interconversion by quenching $^1O_2$ (Stahl and Sies, 1993). Chemical quenching, as opposed to physical quenching of $^1O_2$ by carotenoids leads to the destruction (bleaching) of the carotenoid molecule and the major products are apocarotenals and apocarotenones.

It is noteworthy though that, to date, the majority of the fundamental information regarding $^1O_2$ quenching by carotenoids has been gathered *in vitro*. While experiments in organic solvents and simple micelles and liposomes provide valuable fundamental information on the interactions between carotenoids and $^1O_2$ they do not adequately represent the complex *in vivo* situation. *In vivo* carotenoids rather than being free in solution they are predominantly found associated with protein or lipoprotein structures. The immediate environment of the carotenoid molecule may have a profound effect on its properties and will affect its interaction with other compounds. The extent to which they play a role in protecting cells against $^1O_2$-related biological damage remains unknown (for review see Young and Lowe, 2001).

Carotenoid	N	$K_q/x10^9 M^{-1}s^{-1}$
all trans β-carotene	11	13.0
15-cis-β--carotene	11	11.0
9-cis-β--carotene	11	11.0
a-carotene	10	12.0
Lycopene	11	17.0
Zeaxanthin	11	12.0
Astaxanthin	11 (+2C=O)	14.0
8-apo-β-carotenal	9 (+1C=O)	5.27
Violaxanthin	9	16.0
Lutein	10	6.64

Table 1.4.4-1. Singlet oxygen rate constants for various carotenoids in benzene (from Edge and Truscott, 1999).

1.4.5 Absorption transport and storage of carotenoids

Absorption and metabolism of carotenoids varies among animal species. In humans and only a few other mammals (e.g. the ferret (Liu *et al.*, 2000; Wang *et al.,* 1999b)) an appreciable amount of the carotenoids can be absorbed intact by the mucosal cells and subsequently appear unchanged in the circulation. Thus plasma concentrations in most laboratory animals are normally very low and tissue distribution is not comparable with that in humans. In humans, variable proportions of the carotenoids taken up by the intestinal mucosal cells are metabolised in the process of absorption and this complicates the interpretation of the plasma carotenoid response as an indicator of uptake after administration, when this approach is used in investigations (Parker, 1996).

After uptake by the enterocytes, carotenoids are incorporated into chylomicra and secreted in the lymph, followed by liver uptake and release back into the circulation is association with very low density lipoproteins (VLDL) and ultimately in association with low density lipoproteins (LDL) (Erdman *et al.*, 1993). Carotenoids in the circulation are transported with lipoproteins with a distribution similar to that of cholesterol, so plasma cholesterol concentrations are highly correlated with (and predictive of) circulating carotenoid concentrations (Clevidence and Bieri, 1993; Olson 1994b). Overall approximately 75% of the plasma carotenoids are associated with LDL and the remainder are distributed between VLDL and high density lipoproteins.

Adipose tissue and liver are the major storage depots for carotenoids and high concentrations are found in tissues that are rich in LDL receptors, such as the corpus luteum, adrenal tissue and testes (Woutersen *et al.*, 1999; Rock, 1997).

Differences in the proportionate distribution among isomeric forms have been observed, between the plasma and peripheral tissues for different carotenoids, e.g. β-carotene and lycopene. The amount of all-trans β-carotene greatly exceeds cis-β-carotene in the circulation (>95%), whereas an increased proportion of the cis isomer is observed in tissues.

The proportion of all-trans lycopene in the plasma is <50% of total lycopene and the cis isomers account for more than 79% in prostate tissue (Clinton *et al.*, 1998).

Recently some proteins that bind carotenoids (carotenoproteins) have been identified and characterised in animals and this is expected to shed light on the storage, transport and targeting of carotenoids in animal tissues. This work is complicated by the difficulty of formulating an appropriate aqueous medium for the extraction of such a carotenoid-protein complex. A mammalian β-carotene binding protein of 67 kDa (CCBP) was purified and partially characterised from ferret liver (Rao *et al.*, 1997). In contrast to a molecular size of 67kDa for the mammalian CCBP, the size of carotenoproteins from various lower organisms vary between 18 and 350kDa in their sizes. Alpha-crustacyanine, an astaxanthin-binding protein from the carapace of the lobster is a 350 kDa protein (Keen *et al.*, 1991) and a lutein binding protein isolated and characterised from the silk gland of Bombyx mori larvae (Tabunoki *et al.*, 2002) is a 33 kDa protein. The well-characterised cellular retinoid binding proteins (CRBPs), which have been shown to be essential for vitamin A homeostasis (Ghyselinck *et al.*, 1999) and the cellular retinoic acid binding proteins (CRABPs) are in the 15 kDa range and have been shown to be members of the fatty acid binding proteins (FABPs) (Vogel *et al.*, 2001). Furthermore, the CRBPII and CRABP II proteins have been shown to be transcriptionally controlled through the nuclear receptors, which are the PPARα/RXRα (Suruga *et al.*, 1999) and RARα/RXRα heterodimers (Delva *et al.*, 1999; Bastie *et al.*, 2001), respectively.

1.4.6 Metabolism of β-carotene

β-carotene is the most important provitamin A carotenoid and the most extensively studied so far. Its metabolism in cells can occur via two major β-carotene enzymatic cleavage pathways, the central and/or excentric cleavage pathway.

In the case of central (symmetric) cleavage, β-carotene is metabolised to retinal by β,β-carotene 15,15'-monooxygenase (formerly β,β-carotene 15,15'-dioxygenase (E C.

1.13.11.21)), an enzyme catalysing the central cleavage of β-carotene into two molecules of retinal (cloned and characterised in drosophila (von Lintig and Vogt, 2000), chicken (Wyss *et al.,* 2000), mouse (Redmond *et al.*, 2000) and human (Yan *et al.*, 2001). The produced retinal product can be reversibly converted to retinol by action of members of either the alcohol dehydrogenase (ADH) and/or short-chain dehydrogenase/ reductase (SDR) enzyme families or oxidised to retinoic acid (RA) by the action of members of the aldehyde dehydrogenase (ALDH) family (Duester, 2000). Retinoic acid (RA) is a possible product of β-carotene metabolism (Wang *et al.*, 1991). RA is the active Vitamin A derivative whose pleiotropic effects are transduced by Retinoic Acid Receptors (RARs) and Retinoid X Receptors (RXRs) that are involved in the regulation of gene transcription (Heyman *et al.*, 1992; Mangelsdorf and Evans, 1995; Chambon, 1996).

A recent publication demonstrates that the enzymatic activity of β,β-carotene 15,15'-monooxygenase is regulated by RA by a feedback mechanism in intestinal tissue, but not in lung and liver, of rats and chickens (Bachmann *et al.*, 2002).

In the case of excentric (asymmetric) cleavage of β-carotene two molecules of β-apocarotenal with different chain lengths are formed. An enzyme catalysing exclusively the asymmetric cleavage of β-carotene at the 9'-10', double bond and resulting in the formation of β-apo-10'-carotenal has been recently cloned and characterised in mouse (Kiefer *et al.,* 2001). In contrast to *Drosophila*, both symmetric and asymmetric cleavage pathways exist in vertebrates. For retinoic acid (RA) formation, the β-apocarotenal with the longer chain length can be oxidised to a β-apocarotenoic acid and then shortened, yielding one molecule of RA. For this, a mechanism similar to the β-oxidation of fatty acids has been proposed (Wang *et al.*, 1996). **Fig. 1.4.6-1** shows a representation of the mechanism. Evidence from studies in which the human (Wang *et al.*, 1994) and the ferret (Hebuterne *et al.*, 1995) intestinal tissue liver were employed indicate that 9-cis-β-carotene functions as a precursor of 9-cis retinoic acid, a retinoid with strong affinity to both RARs and RXRs whereas the all trans isomer has a selective affinity for RARs (see subsection 1.3.8). Liver cytochromes P450 have been shown to take part in the metabolism of retinol and retinoic acid to polar metabolites (White *et al.*, 1997), so the possibility of the involvement of that system in carotenoid metabolism

is feasible. Overall, the conversion of β-carotene to vitamin A appears to be tightly regulated through vitamin A homeostasis (Olson, 1994a). It is dependent on the food matrix (De Pee, *et al.*, 1998), food processing (Rock *et al.*, 1998), fat intake (Takyi, 1999), protein intake and vitamin A status (Ribaya-Mercado *et al.*, 2000). It has been demonstrated that the conversion of β-carotene to vitamin A diminishes inversely to the intake of β-carotene (Brubacher and Weiser, 1985; Olson 1994b), so skin can yellow with excessive dosing (hypercarotenemia) and no symptoms of hypervitaminosis A are associated with hypercarotenemia (Dawson, 2000).

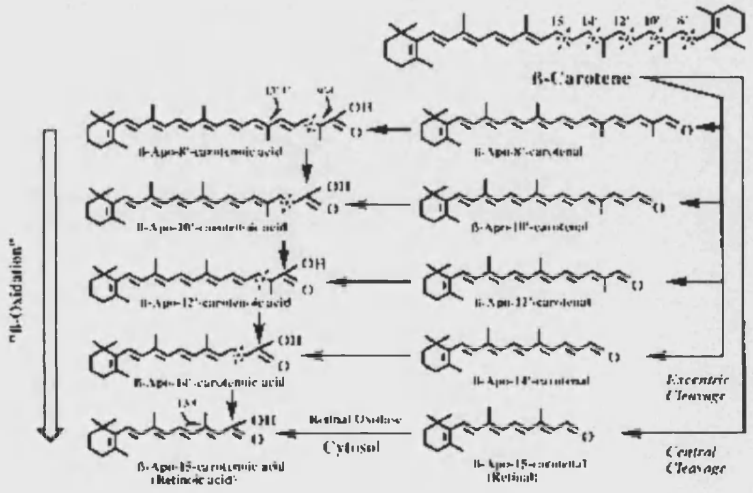


Fig. 1.4.6-1. The central and excentric cleavage of β-carotene (reproduced from Wang *et al*, 1996)

1.4.7 Lycopene and metabolism

Lycopene is one of the predominant carotenoids in human plasma (Rock, 1997). It is an acyclic carotenoid with 11 linearly arranged conjugated bonds and two non conjugated double bonds and the most potent $^1O_2$ quencher of all the carotenoids (DiMascio *et al.*, 1989). Lycopene lacks the β-ionone ring structure and is devoid of provitamin A activity. The enzyme catalysing the asymmetric cleavage of β-carotene at the 9'-10' double bond is active with lycopene as well, in a reaction producing apo-lycopenals of different chain lengths (Kiefer *et al.*, 2001).

In studies aimed at determining whether lycopene or a compound shown to be produced by the oxidation of lycopene (Kim *et al.*, 2001), namely acyclo-retinoic acid (the open chain analog of RA), would activate RARs it was shown that it could activate RARs with a 100-fold lower potency than retinoic acid (Ben-Dor *et al.*, 2001).

1.4.8 Functions and biological activities of carotenoids

The antioxidant actions of β-carotene have been demonstrated in many studies (Woodall *et al.*, 1997; Krinsky, 1993) and many studies support the fact that β carotene exhibits protective effects against lipid peroxidation resulting from free radicals or singlet oxygen. This occurs in organic solution, liposomes, liver microsomes and cells in culture. The antioxidant effect of carotenoids in LDL has been suggested to be the biological link between higher level of dietary carotenoids and reduced risk for cardiovascular disease (Krinky, 1994). However *in vivo* this relation between carotenoids and LDL oxidation has not been consistent. A biological explanation is that the presence of a-tocopherol in LDL is of greater importance in the prevention of LDL oxidation (Krinsky, 2001; Rock, 1997; Young and Lowe, 2001).

Carotenoids react rapidly with oxidising agents and free radicals and at the end of the reaction carotenoid products have been detected in small quantities and most of the

carotenoid has disappeared. The products depend on the oxidising conditions, arising from a variety of oxidation reactions that affect different parts of the molecule (cleavage of the polyene chain, epoxidation of endocyclic double bonds or reactions at allylic positions). Mechanisms proposed for these reactions include the addition of peroxyl radicals to the polyene chain, electron capture from the carotenoid, and hydrogen abstraction from allylic positions (Woodall *et al.*, 1997; Burton and Ingold, 1984). Although most work on the antioxidant actions of carotenoids has been carried out with β-carotene studies comparing carotenoids also exist.

In a study of Woodall and associates carried out in solution, all carotenoids reduced lipid peroxidation of egg yolk phosphatidyl choline (EYPC) from 2,2'-azobis(2,4'-dimethylvaleronitrile (AMVN)-derived peroxyl radicals and a clear correlation between inhibition of lipid peroxidation and destruction of the carotenoid was observed for all carotenoids apart from lycopene. Lycopene was found to react more rapidly than the other carotenoids, but this was not associated with increased antioxidant activity. When the carotenoids were incorporated in liposomes the results were similar in that they exhibited antioxidant activity, but the order of potency of the carotenoids was different and the level of destruction of the carotenoids was much lower than in solution. The effects of the carotenoids regarding membrane protection were different when they were tested against AAPH induced oxidation (production of peroxyl radicals in the aqueous phase) the carotenoids that afforded the least protection were β-carotene or lycopene However both β-carotene and lycopene would be expected to be restricted to the inner core of the membranes and the collision rates with peroxyl radicals in the aqueous phase would be expected to be very low (Woodall *et al.*, 1997).

The antioxidant activity of β-carotene and other carotenoids has been suggested to shift into a prooxidant activity depending on the redox potential of the carotenoid molecule as well as the biological environment in which it acts. The prooxidant potency of carotenoids is influenced by several factors, including pO_2, carotenoid concentration and interaction with other antioxidants (for review see Palozza, 1998 and Young and Lowe, 2001). The procarcinogenic effects of oxidation products (typically epoxides but also apocarotenals) of carotenoids has been proposed. For example the binding of benzo[a]pyrene to DNA is promoted in the presence of oxidation products of β-

carotene, but inhibited in the presence of β-carotene itself (Salgo *et al.*, 1998). Elevated levels of such oxidative products have been observed in vitro in the lungs of tissues from smoke-exposed ferrets compared to controls. High levels of these products have been shown to downregulate the RARβ-gene (Liu *et al.*, 2000; Wang and Russell, 1999). In the presence of H_2O_2 or xanthine/xanthine oxidase the ability of β-carotene and lycopene to protect against DNA damage (single strand breaks as measured by the Comet Assay) in HT29 adenocarcinoma cells, was inversely related to carotenoid dose (Lowe *et al.*, 1999). Relatively high doses of β-carotene also failed to protect against H_2O_2 and tertbutyl hydroperoxide induced DNA damage in HepG2 cells, as measured by the Comet assay (Woods *et al.*, 1999). It is known that different carotenoids influence the properties of membranes into which they are incorporated in a different manner but also that their effectiveness against ROS in the aqueous and lipid phase is quite different (Woodall *et al.*, 1997a; 1997b).

β-carotene and lycopene are derived from plants and they share similar initial synthetic pathways with cholesterol, which is synthesized in animals but not in plant cells (Stahl and Sies, 1996; Fuhrman, 1997). The investigators examined the effect of the carotenoids on macrophage cholesterol metabolism. The cellular cholesterol synthesis from acetate was suppressed. Interestingly, lycopene and β-carotene augmented the activity of the macrophage receptor similarly to the effect of lovastatin. The authors concluded that dietary carotenoids may act as moderate hypocholesterolemic agents, secondary to their effects on macrophage 3-hydroxy-3 methyl-glutaryl coenzyme A reductase, the rate limiting enzyme in cholesterol biosynthesis (Fuhrman *et al.*, 1997).

Anticarcinogenic activities have been demonstrated for both provitamin A and nonprovitamin A carotenoids in animal models (Krinsky, 1993). However, no animal experiments had been reported on the effect of high doses of BC on lung carcinogenesis prior to the human intervention trials. An arising question is whether high doses of BC fed to experimental animals reach the target tissues. This problem was corrected by the introduction of the ferret as an experimental animal (Liu *et al*, 2000, Wang *et al.*, 1999b; Wolf, 2002).

Several studies reported antiproliferative effects of lycopene against cancer cells in culture (Livny *et al.*, 2002; Nara *et al.*, 2001; Karas *et al.*, 2000; Ben Dor *et al.*, 2001, Prakash *et al.*, 2001) by interfering with growth factor receptor signalling and cell cycle progression (Levy *et al.*, 1995) or by enhancing gap-junctional communication (Livny *et al.*, 2002). In several types of cell cultures β-carotene, lycopene as well as other carotenoids have been shown to promote inhibition of malignant transformation. The prevention of malignant transformation in mouse fibroblast cells is found to be mediated by increased gap junctional communication between cells, and this was shown to be due to the up-regulation of the connexin 43 gene (Bertram, 1991; Bertram and Bortkiewicz, 1995). These effects were observed in response to both provitamin A and non-provitamin A carotenoids, with using concentrations in the cell culture medium that are achievable by increased dietary intake. Other investigators examined the ability of canthaxanthin to increase the expression of a reporter gene driven by the RAR-β promoter. The gene was activated, and an active retinoid (4-oxoretinoic acid) that was shown chemically to be produced from canthaxanthin. (Nikawa *et al.*, 1995). Also acyclo retinoic acid (the open chain analog of RA) has been shown to activate RARs, however, with a 100-fold lower potency than retinoic acid (Ben-Dor *et al.*, 2001).

Carotenoids have also been shown to exert effects on cell differentiation. These compounds were, however much less active than retinoic acid, which is now used clinically to induce differentiation in promyelocytic leukaemia. The potential clinical relevance of a relationship between carotenoids and immune responses is illustrated by the observation that β-carotene supplementation could prevent the photosuppression of delayed type hypersensitivity (Fuller *et al.*, 1992; Herraiz *et al.*, 1998). β-carotene and other carotenoids have been shown to enhance the release of ILα and TNFα from glycogen induced murine peritoneal adherent cells (Okai and Higashi-Okai, 1996). Incubation of human peripheral blood mononuclear cells for 72h with β-carotene or canthaxanthin at 10^{-8} mol/L in EtOH increased the number of cells expressing markers for natural killer cells and IL-2 receptor. Similar changes were seen after exposure to retinoids (for review see IARC Working Group for the Evaluation of Cancer, 1998).

1.4.9 Carotenoids in chemoprevention and treatment

β-carotene supplementation is recognised as an effective treatment modality in the management of the genetic photosensitivity disease, erythropoietic porphyria. The treatment is based on the function of carotenoid pigments in plants and microorganims. Erythropoietic porphyria is characterised by defective ferrochelatase, the enzyme that inserts iron into protoporphyrin to produce heme, and this results in an accumulation of protoporphyrin. Presumably the accumulation of carotenoids in the tissue [skin] prevents photosensitization by the porphyrins (Mathews -Roth, 1993).

β-carotene plasma levels are a result of dietary intake and the range among different individuals is very large. The means of circulating levels in European and American populations have been reported from several studies and are in the range of 0.17-2.25 μM (Armstrong *et al.*, 1997; Heseker *et al.*, 1991; The α-tocopherol and β-carotene study group (ATBC), 1994; Omenn *et al.*, 1996, Carotene and Retinol Efficacy Trial (CARET); Hennekens *et al.*, 1996, Physicians Health Study (PHS)). The tissue levels for β-carotene are shown on **Table 1.4.9-1.**

As previously reviewed (Mayne, 1996; Peto *et al.*, 1981; Block *et al.*, 1992; Ziegler, 1989) epidemiological evidence has strongly linked higher levels of circulating carotenoids with reduced risk of many cancers, cardiovascular disease, cataracts and macular degeneration. On the basis of the substantial epidemiological evidence linking carotenoids to reduced chronic diseases especially lung cancer and on the basis of basic and experimental supportive evidence, several large clinical trials were initiated to test the effect of intervention. Six large multicenter clinical trials were conducted to test the effect of the intervention with β-carotene (ATBC study group, 1994; Omenn *et al.*, 1996 [CARET]; Hennekens *et al.*, 1996 [PHS], Greenberg *et al.*, 1990, Greenberg *et al.*, 1994; Blot *et al.*, 1993) and failed to confirm this association in well nourished populations (Cook *et al.*, 2000; Rock *et al.*, 1997; Bertram, 1999; Wang *et al.*, 1999b). The results of the major intervention trials are summarised on **Table 1.4.9-2.** It has been suggested that the difference between observational and intervention studies regarding β-carotene may be a consequence of the use of different populations with varying nutritional status and smoking habits, different tumor sites, combination

supplements vs single agent supplements and perhaps even different formulations. Furthermore, dietary blood or tissue carotenoids might primarily serve as a marker of other protective factors and β-carotene may act synergistically with other compounds found in a natural food matrix, and finally, β-carotene may have been administered too late in the carcinogenic process (Mayne, 1996). In the intervention trials the load of β-carotene was similar, but the resulting average plasma levels after supplementation differed substantially (ATBC 5.5 μM; CARET 3.9 μM; PHS 2.2 μM; Linxian: 0.86 μM). Understanding the mechanisms related to high dose β-carotene supplementation is important, due to a continuing interest in the potential of β-carotene as a chemopreventive agent (Liu, 2000).

Serum concentrations of lycopene in humans range from 0.01-1.9 [PBNI2]μM although interindividual variation is very large with standard deviations in the range of 50% of the mean or more (Gerster, 1997; Campbell *et al.*, 1994; Brady *et al.*, 1996; Ribaya-Mercado *et al.*, 1995; Stahl *et al.,* 1992; Carughi *et al.,* 1994; Micozzi *et al.,* 1992; Peng *et al.*, 1995; Clinton *et al.*, 1996).

Increased levels of lycopene in the diet and in plasma have been associated with decreased risk of prostate (Giovanucci *et al.*, 1995; Giovanucci, 1999; Lu *et al.*, 2002) and cervical cancer (Kanetsky *et al.*, 1998).

Protective effects of lycopene towards oxidative stress mediated damage of the skin were suggested following a study on carotenoid levels in human skin upon irradiation with UV light. When skin was subjected to UV light stress, more skin lycopene was destroyed than β-carotene. Because carotenoids are consumed in the process of radical quenching, a preferential protective role of lycopene has been suggested (Ribayo-Mercado *et al.*, 1995). Also, lycopene when supplemented as tomato paste with olive oil has also been shown to protect against UV induced erythema after a 10-week supplementation (Stahl *et al.*, 2002). The tissue levels of lycopene are shown on **Table 1.4.9-1.**

Tissue	β-carotene (nmol/gwet weight)	Lycopene (nmol/gwet weight)
Liver	3.02 (Stahl and Sies, 1996) 1.82 (Kaplan, 1990)	1.28 (Stahl and Sies, 1996) 2.45 (Kaplan, 1990)
Kidney	0.55 (Stahl and Sies, 1996) 0.31 (Kaplan, 1990)	0.15 (Stahl and Sies, 1996) 0.39 (Kaplan, 1990)
Adrenal	5.60 (Stahl and Sies, 1996) 9.39 (Kaplan, 1990)	1.9 (Stahl and Sies, 1996) 21.60 (Kaplan, 1990)
Testes	2.68 (Stahl and Sies, 1996) 4.36 (Kaplan, 1990)	4.34 (Stahl and Sies, 1996) 21.36 (Kaplan, 1990)
Ovary	0.45 (Stahl and Sies, 1996) 0.97 (Kaplan, 1990)	0.25 (Stahl and Sies, 1996) 0.28 (Kaplan, 1990)
Adipose	0.38 (Stahl and Sies, 1996) 0.38 (Kaplan, 1990)	0.20 (Stahl and Sies, 1996) 1.30 (Kaplan, 1990)
Lung	0.22 (Nierenberg and Nann, 1992)	
Skin	0.27 (Nierenberg and Nann, 1992) 1.41 (Ribaya-Mercado *et al.*, 1995)	0.42 (Nierenberg and Nann, 1992) 1.60 (Ribaya-Mercado *et al.*,1995)
Prostate	0.09-1.7 (Clinton *et al.*, 1996)	0-2.58 (Clinton *et al.*, 1996)

Table 1.4.9-1 Tissue concentrations of β-carotene and lycopene

Study	Duration	Participants	Daily Supplementation	End-Points	Outcome
Greenberg et al., 1990	5 yrs	1805 patients with a history of skin cancer	50mg βc vs. placebo	Occurrence of new basal or squamous-cell carcinoma	No effect
Greenberg et al., 1994	5-8 yrs	Patients with a history of adenomas	25mg βc +/- 1g VitC and 400mg VitE	Occurrence of colorectal adenoma	No effect
ATBC, 1994[AU3]	5-8 yrs	29133 male smokers	20mg βc +/- VitE) vs. Placebo	Incidence of lung cancer	18% increase in incidence of lung cancer in smokers, 8% increase in total mortality
Omenn *et al.*, 1996 [CARET]	4yrs Halted	18314 -male smokers -former smokers -persons exposed to asbestos	30mg βc + 25,000 IU VitA Vs. placebo	Incidence of lung cancer CVD	28% increase in incidence of lung Cancer, 26% increase in CVD 17% increase in mortality
Hennekens *et al*, 1996 [PHS]	12yrs	22071 healthy male physicians	50mg βc every other day	Incidence of malignant neoplasms, CVD	No effect
Blot *et al.*, 1993 [LINXIAN]	5-6yrs	Undernourished adults	50mg βc +50μg selenium +30mg Vit E vs other vitamins	Incidence of cancers of the upper GI tract	Reduction

Table 1.4.9-2 Table summarising the results of the major intervention trials with β-carotene. Abbreviations used in Table, βc: β-carotene; CARET: Carotene and Retinol Efficacy Trial; ATBC: α-tocopherol and β-carotene study; PHS: Physicians Health Study; CVD: cardiovascular disease; GI; gastrointestinal.

1.5 Nuclear Receptors (RARs, RXRs, PPARs)

Nuclear receptors comprise a superfamily of ligand activated transcription factors that regulate the expression of genes involved in the development and adult physiology (Mangelsdorf and Evans, 1995). They provide multicellular organisms with a means to directly control gene expression in response to a wide range of developmental, physiological and environmental stimuli (Giguere, 1999).

Retinoic acid receptors are members of the nuclear receptor superfamily that includes the 9-cis retinoic acid receptors (RXR α,β,γ) and orphan receptors. Orphan receptors are nuclear receptors whose cognate ligands are (or were) unknown and have been the subject of intense study over the past decade. Retinoic Acid Receptors (RAR α, β, γ), Peroxisome proliferator activated Receptors (PPAR α, γ, δ), Chicken ovalbumin upstream promoter transcription factor (COUP) and Nerve Growth Factor Receptor Induced (NGFI-B) belong to the orphan receptor family. Upon ligand binding nuclear receptors modulate gene expression.

Retinoid response pathways are mediated by two classes of receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (Zhang *et al.*, 1992; Kastner *et al.*, 1997). RXRs can bind to DNA both as homo- and heterodimers and the homodimers are induced exclusively by 9-cis retinoic acid. RXRs are major regulators of gene expression and serve as a common heterodimeric partner for RARs, PPARs, liver receptors (LXRs) and numerous other nuclear receptors (LeBlanc and Stunnenberg, 1995; Giguere, 1999). 9-cis RA is a ligand for both RARs and RXRs and all-trans RA is a ligand for RARs only (Mangelsdorf and Evans, 1995).

The PPAR subfamily includes three isoforms (α, β or δ, and γ) involved in the maintenance of lipid and glucose homeostasis. All PPAR isoforms bind to PPAR response elements (PPRE) as a heterodimer with RXR located in the promoter of target genes (Schoonjans *et al.*, 1997, Shi *et al.*, 2002, Chinetti *et al.*, 2000). In the PPAR/RXR heterodimer, both receptors are independently responsive and synergistically activated in the presence of both ligands (Desvergne and Wahli, 1999).

Several studies have demonstrated that PPARα regulates fatty acid uptake, activation into acyl-CoA esters, and β- and ω-oxidation of fatty acids. Natural ligands for PPARα include among others eicosanoids derived from arachidonic acid (for review see Reddy and Hashimoto, 2001; Chinetti *et al.*, 2000). Among the naturally occurring eicosanoids15-deoxy-Δ-^{12,14}-prostaglandin J_2 (PGJ2) is the compound with highest affinity on PPARγ. PPARγ has been shown to play a central role in activating adipogenesis both *in vitro* and *in vivo* (Tontonoz *et al.*, 1994) and to play a role in the development of the atherogenic lesion (Kliewer, 1999). The PPARδ isoform is ubiquitously expressed at moderate levels in humans with a higher expression in the placenta and the large intestine. PPARδ is related as well as PPARα with increased expression of fatty acid binding proteins (FABPs) and the cellular binding proteins (CRBP) (for review see Desvergne and Wahli, 1999).

It has been demonstrated that nuclear hormone receptors as well AP-1, basic-loop – helix factors, STATs, cAMP response element-binding protein, and nuclear factor-κB (NF-κB) utilise the CREB binding protein (CBP) or its homolog (p300) to function as a nuclear receptor cofactor. CBP/p300 are viewed as integrators of multiple signalling pathways, linking membrane receptor signalling and nuclear receptor activation pathways, as well as being a key limiting factor for which all the above mentioned pathways must compete (for review see Goodman and Smolik, 2000; Desvergne and Wahli, 1999).

Non-liganded receptors may associate with transcriptional co-repressors such as silencing mediator for RARs and TRs (SMRT) and nuclear receptor co-repressor (NcoR) resulting in suppression of basal transcriptional activity (Nagy *et al.*, 1997; Chen and Evans, 1995; Sande and Privalsky, 1996). Thus, hormone dependent dissociation of SMRT from a RXR-RAR heterodimer displaces the mSin3 /HDAC1 complex from a chromatin bound complex (Nagy *et al.*, 1997). Phosphorylation is one potential candidate for mediating receptor function by such a pathway. Another mechanism by which receptors can modulate gene expression is by cross-coupling to augment or inhibit signalling pathways mediated by other classes of transcription factors. A very good example, is the inhibition of AP-1, by retinoids, thyroid hormones and glucocorticoids (Beato *et al.*, 1995).

Evidence exists suggesting that carotenoids including β-carotene can upregulate expression of retinoic acid receptors in various cell types (Ponnamperuma *et al.*, 2000; Ben-Dor *et al.*, 2001; Liu *et al.*, 2000; Wang *et al.*, 1999b).

RARα and RXRγ transcripts, the main nuclear retinoid receptors in human skin, are essentially absent in undifferentiated squamous cell carcinomas in man. UV induced upregulation of AP-1 activity and AP-1 driven matrix degrading metalloproteinase genes can be inhibited in human skin by pretreatment of skin with retinoic acid (RA). UVB irradiation at 2MED (twice minimal erythema dose) causes a rapid reduction of RARα and RXRγ, at protein and mRNA level and a near total loss of RA induction of the RARα and RXRγ induced genes cellular retinoid acid binding protein II (CRABP-II) and RA 4-hydroxylase (Wang *et al.*, 1999a).

1.6 Special features of carotenoid work

As carotenoids are less stable than many other substances, precautions and special procedures must be used to minimise the risk of degradation and formation of artefacts. In particular exposure to oxygen, heat, light, acid and in some cases bases must be avoided whenever possible (Britton, 1995; IARC Working Group for the Evaluation of Cancer, 1998). All operations should be carried out in an inert atmosphere (Nitrogen or Argon), at low temperature (room temperature, about 20^0C) in the dark or diffuse light, under acid free conditions and peroxide free solvents. The usual indication of carotenoid breakdown is bleaching, i.e. loss of the colour, due to breakage of the chromophore. A variety of breakdown products have been detected, most often apocarotenoids of various chain lengths, produced by cleavage of any of the double bonds in the polyene chain (Palozza and Krinsky, 1992b; Liebler, 1993). Similar products are derived from the chemical reaction between carotenoids and singlet oxygen or other oxidising agents.

Geometrical (cis-trans) isomerisation occurs readily when carotenoids are exposed to factors such as heat and light (Britton, 1995). As most carotenoids of interest are highly lipophilic and they are virtually insoluble in water, supplying these compounds in a

bioavailable form has been a major problem. This has been achieved in several ways. Tetrahydrofuran (THF) has been widely used as a solvent since its introduction for this purpose (Bertram *et al.*, 1991). If suitable precautions are taken, this solvent is not toxic and produces a pseudo-emulsion with high bioavailability (Cooney *et al.*, 1993). A second successful method of delivery involves incorporating carotenoids in liposomes. Here too adequate uptake of carotenoids into cells has been demonstrated. An increasing number of carotenoids are also available commercially in the form of beadlets composed of a protein and carbohydrate matrix containing micro-dispersed carotenoid in oil-control beadlets are also available, containing all of the packaging but no carotenoid. (the principal disadvantage of these beadlets is that they contain 90% packaging material that includes antioxidants).

1.7 Aims and Objectives

The aim of this study is to investigate the modulation of the level of UVA induced transcriptional activation of heme oxygenase 1 (HO-1) by β-carotene and lycopene. Previous studies have shown that the UVA induced HO-1 gene activation occurs via singlet oxygen mediated pathway. Carotenoids are potent singlet oxygen quenchers and they are expected to suppress the UVA induced HO-1 mRNA accumulation.

Objectives

Experiments were designed to investigate whether cultured FEK4 cells take up intact (the lipophilic and oxygen labile) all-trans-β-carotene and to select an appropriate method for the delivery of β-carotene to FEK4 cells in culture.

In order to determine this, the time kinetics of β-carotene uptake by FEK4 cells, its stability in the growth medium of cultured FEK4 cells in different growth phases (exponentially growing, confluent and growth arrested cells), as well as the time kinetics and the level of uptake of lycopene by exponentially growing FEK4 cells and the isomerisation pattern of lycopene in cells and medium will be monitored.

As detailed in the introduction, there is evidence that specific isomers and metabolites of β-carotene might be involved in the biological activity of the carotenoid, so the level of isomerisation, retinol and apocarotenal formation in cells will be monitored in sham and UVA irradiated cells.

Published literature is consistent with the notion that carotenoids (including β-carotene) can promote health when taken at dietary levels, but may have adverse effects when taken in high dose.

In order to establish the modulation of UVA (100 and 250 kJ/m^2) induced HO-1 mRNA by β-carotene, the cells will be incubated with a large range of concentrations of the carotenoid in the cell culture medium prior to irradiation. This will be examined in detail and UVA dose response experiments will be carried out following incubation of FEK4 cells with a single concentration of β-carotene in the medium.

The experiments with lycopene will be based on the same principles.

We also plan to test the modulation of UVA induced HO-1 mRNA levels in different growth phases of FEK4 cells, since especially growth arrested cells resemble more the state in which dermal fibroblasts are found in human skin.

Real Time Reverse Transcription Polymerase Chain Reaction will be developed to replace the Northern Blot Analysis for measurements of the ratio of HO-1 mRNA versus GAPDH in experimental samples, as this is a very sensitive technique.

2 MATERIALS AND METHODS

2.1 Chemicals and Reagents.

All the chemicals used were supplied by BDH, Dorset, UK., except for:

- 99% all-trans-β-carotene and 96.7% all-trans lycopene (provided by Hoffman-La Roche, Switzerland).
- Tetrahydrofuran (THF 99.7% containing 0.025% BHT), Ethanol (99.7-100%) and (3-[N-morpholino] propanesulphonic acid) (MOPS) (Sigma, Aldrich, UK).
- Foetal calf serum (PAA laboratories, GmbH, Linz, Austria).
- Phosphate Buffered Saline (PBS) (Oxoid, Basingstoke, UK).
- ^{32}P dCTP (Amersham, Buckinghamshire, UK).
- Sodium carbonate, Glutamine, Penicillin and Streptomycin, Trypsin, Trizol reagent, oligo (dT_{12-18}) primers, primers P1, P2, P3, P4, G1, G2, R1 and R2 (5 μM), Platinum *Taq* DNA polymerase, SuperScript II RNase H^- Reverse Transcriptase and SuperScript First Strand Synthesis System for RT-PCR (Gibco BRL, Life Technologies, Scotland-Invitrogen, Paisley, UK).
- SYBR Green I containing Taq DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye, and 10mM $MgCl_2$ (Roche Diagnostics, East Sussex, UK).
- *Hin*d III (10 000 units/ml), *Eco*R I (60 000 units/ml) (Promega, UK).

2.2 Cell culture

The normal human skin fibroblast cell line FEK4 (Tyrrell and Pidoux, 1986) was derived from a foreskin biopsy and cultured routinely in Earle's modified minimal essential medium (EMEM) supplemented with 15% (unless otherwise noted) foetal calf serum (FCS) (inactivated at 56°C for 45 minutes), glutamine (2mM), sodium bicarbonate (0.2% w/v), penicillin and streptomycin (50 IU/ml each). Cells were passaged by trypsinization once a week and were used for experiments between passages 11 and 13. Three different growth phases of FEK 4 were employed in our experiments (described as follows) in order to determine if this would affect the experimental outcome. In all experiments, cell monolayers were UVA irradiated after a three-day incubation period with the β-carotene solutions.

Growth arrested FEK4 monolayers. As previously described (Tyrrell, 1984) fibroblasts were seeded at a density of $3X10^5$cells per 10cm dish in a 15%FCS growth medium. After they reached a confluency of approximately 60% (2.5 days), the medium was removed, medium traces washed off with PBS and a 0.5% FCS cell culture medium was introduced to the dishes. Cells were incubated for a further week prior to the addition of the β-carotene solutions to the conditioned medium.

Confluent FEK4 monolayers. Fibroblasts were seeded at a density of $3.5X10^5$ per 10cm dish and grown to 100% confluency in four days prior to the introduction of the appropriate β-carotene solutions to the cell culture medium.

FEK4 in exponentially growing phase. The β-carotene or lycopene in THF solutions were added at initial seeding. For experiments with β-carotene the seeding density was $3.5X10^5$ cells per 10cm dish and cell monolayers were UVA irradiated after a 3.5 day incubation period, when the FEK4 monolayers were 85-95% confluent. For experiments with lycopene the seeding density was $4X10^5$ cells per 10cm dish and cell monolayers were UVA irradiated after a 3.5 day period.

Carotenoid solutions. The β-carotene or lycopene 2mM stock solutions in THF and appropriate dilutions of the stock solution were prepared in a glove box under Argon, gas–tight sealed and frozen (-80^{0}C) to be used for experiments within a week.

A wavelength scan of each carotenoid in THF determined that the λ_{max} for β-carotene is 459nm and 479nm for lycopene in this solvent and that the range of concentrations that can be measured with this method is 2-5μM.

After appropriate dilutions, spectrophotometry was performed to confirm the concentration of each β-carotene or lycopene solution to be added to the cell cultures (molar extinction coefficients in THF ε 135110 cm^2/mol, λ_{max} 459 for β-carotene **(Fig. 3.1.4-1)** and ε 212620 cm^2/mol, λ_{max} 480 for lycopene **(Fig. 3.1.4-2)**).

2.2.1 Delivery of carotenoids to the cultured cells.

In order to evaluate the best method for introducing β-carotene into FEK 4 cell cultures two methods were initially tested for their efficacy in delivering intact all-trans-β-carotene into the cells. The delivery systems were compared using either THF or THF/EtOH (1:1) and were tested beforehand for cytotoxicity (trypan blue dye exclusion assay). Neither of the solvents was found to be toxic to the cells at concentrations in the cell culture medium ≤0.5% for THF and ≤1% for THF/EtOH. Experiments were performed with final concentrations 1.5 and 4.5μM β-carotene in the medium with the two carrier systems. At the selected intervals, (2.5, 5, 24, 48, 72h) the cellular content in β-carotene was evaluated by HPLC analysis (Hoffman-La Roche laboratories) (see Appendix 1.1 for a description of the HPLC methodology used). Cell pellets were dispatched to Hoffman La Roche after thorough washing of the cell monolayers (Phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA) 6x5min at 37^{0}C). This washing protocol was shown to be the most effective in preventing β-carotene from sticking to the outer surface of cells in preliminary experiments (unpublished results, R.G., Basel, Switzerland). The results of experiments performed to

compare the effectiveness of 6 different washing protocols are described in detail in Appendix 2.

Following the washing step the cells were detached from their plates by trypsinisation, pelleted and the cell pellets frozen (-80^{0}C). In parallel, the stability of β-carotene in the growth medium under cell culture conditions was monitored (over the same period of time and at the same time intervals).

After the evaluation of the two methods for delivery of carotenoids to FEK4 cells THF was used as a delivery system of β-carotene and the cell monolayers were thoroughly washed (PBS+2%BSA 6x5min at 37^{0}C) before UVA (or sham) irradiation. After the first experiments on modulation of UVA HO-1 mRNA by β-carotene that did not give reproducible results (results not shown) and in order to reduce a possible source of error, the final concentration of THF (containing 0.025% BHT) was 0.3% in all cell culture dishes of all experiments. The optimum time for introducing β-carotene to the cell culture for the purposes of this study proved to be at initial seeding, as the levels of HO-1 mRNA induction by UVA were lower at later times.

The protocol used for the delivery of β-carotene to FEK4 cells was used also for the delivery of lycopene to FEK4 cells. The protocol used for the HPLC analysis of content of medium samples and cell pellets in all-trans- and cis-lycopene is described in detail in Appendix 1.2.

2.3 UVA source and irradiation conditions

Cell populations grown at 85-100%confluency were irradiated using a UVASUN 3000 lamp (Sellas, Munich, Germany), which emits broad-spectrum UVA light. The UVA dose was measured using an IL 1700 radiometer (International Light, Newbury, RI, USA). Irradiation was carried out to give total UVA doses of 50, 100, 200 and 250kJ/m^2 at a controlled room temperature of 25^{0}C.

Prior to irradiation, medium was removed and retained. The cell monolayers were washed 6X5min with PBS+2%BSA at 37^{0}C and finally rinsed with PBS. Cells were UV- or sham irradiated in PBS supplemented with Ca^{2+}/Mg^{2+} (0.01% each). After irradiation the original medium was added back and the cells incubated for 4-4.5h. The total time during which the cells were in PBS was 1.5-2 h prior to readdition of the original media.

2.4 Measurement of UVA induced HO-1 mRNA accumulation

Expression of the human HO-1 gene is induced by UVA at levels that permit modulation by carotenoids to be monitored. THF with 0.025% BHT was used as a delivery system for β-carotene and lycopene and for all experiments the cell monolayers were thoroughly washed (PBS+2%BSA, six times for 5 min) before UVA irradiation. The optimum time for introducing the carotenoids to the cell culture proved to be at initial seeding.

Total RNA was extracted from cells by the one step acid guanidinium thiocyanate/phenol/chloroform method or with Trizol® (Chomczynski and Sacchi, 1987). The cells were lysed in 4 M guanidinium thiocyanate solution (0.5% sodium N-laurylsarcosine, 25 mM sodium citrate (pH 7.0) and 0.1 M β-mercaptoethanol. Protein extraction was done with water-saturated phenol and chloroform: isoamyl alcohol (49:1). RNA was redissolved in RNase -free water and quantified by absorption spectrophotometry. The HO-1 mRNA was measured either by Northern Blotting, Taqman or by Real Time PCR (LightCycler).

2.4.1 Northern Blotting

First 10μg/sample total RNA was electrophoresed on a 3-[N-morpholino] propanesulphonic acid (MOPS)/formaldehyde agarose (1.3%) gel and transferred to a Zeta-Probe (Biorad, Hercules, CA, USA) nylon membrane by capillary blotting. After transfer, membranes were baked for 2 h at 80^0C, prehybridised and hybridised at 68^0C. Prehybridisation and hybridisation solutions were 7%SDS, 250 mM $NaHPO_4$, 1mM ethylenediamineacetate (EDTA). The HO-1 cDNA probe used was a 1000bp *Eco* RI fragment of the human heme oxygenase clone 2/10 (Keyse and Tyrrell, 1989). The glyceraldehyde phosphate dehydrogenase (GAPDH) GAPDH cDNA probe was a 1400bp *Hind III/ Eco* RI fragment of the rat GAPDH gene. cDNA probes were ^{32}P-labelled using a high prime enzyme kit (Boehringer Mannheim) according to manufacturers instructions, the labelled cDNA probe was then purified using Elutip-D column (Schleider and Schuell, Germany). Membranes were pre-hybridised and hybridised in a 7% w/v SDS, 250mM Na_2HPO_4, 1mM ethylenediaminetetraacetate (EDTA). The ^{32}P-labelled HO-1 probe was added to the hybridisation solution. After hybridisation non-specifically bound probe was washed from the membrane using SDS (at a final concentration of 0.1% w/v) and 20X SSPE (3M NaCl, 0.2M Na_2HPO_4, 20mM EDTA, pH 7.4). Washings were performed twice with 6X SSPE for 5 minutes at 37°C, twice with 1X SSPE for 15 minutes at 37°C and twice with 2X SSPE for 15 minutes at 68°C. The membranes were sealed in a plastic bag and exposed to a phosphorimager screen. After autoradiography, the HO-1 cDNA probe was stripped from the membrane by boiling in 0.3% w/v SDS (twice for 30 min each time), and blots were re-probed with the GAPDH cDNA probe for an internal control of loading between samples. RNA levels were quantified using a phosphorimmager (Molecular Dynamics, Sunnyvale, CA, USA) and use of the software; Image Quant 3.3.

2.4.2 Reverse transcriptase (RT)- PCR

RT-PCR represents a sensitive and powerful method for RNA analysis (Freeman *et al*, 1999; Lie and Petropoulos; 1998, Ahmed *et al.*, 1995) since it enables the detection and measurement of very low levels of mRNA, which are normally below the detection

limit of other conventional methods. However, many errors could be introduced in the mRNA quantitation by RT-PCR. The RT-step is the source of most variability in a quantitative RT-PCR experiment. The reverse transcriptase is sensitive to salts, alcohols or phenol remaining from the RNA isolation. Inter-tube and inter-experiment variations are common in RT reactions (Freeman *et al.*, 1999). For compensating for the variability of the efficiency of the reverse transcriptase reaction a reference gene can be assayed together with the unknown target and their final ratio evaluated (Orlando *et al*, 1998). For relative quantification of RNA it is possible to use a DNA standard curve. Doing this requires the assumption that the reverse transcription of target genes is the same in all samples, but the exact efficiency of this process does not need not to be known. Following the RT reaction, the cDNA of target genes is amplified by PCR. We initially aimed to set up the conditions for competitive PCR quantitation of HO-1 mRNA levels as previously described (Ahmed *et al.*, 1995), but at the same time we had the opportunity to use Real Time PCR, a method that is increasingly used after reverse transcription (RT) for mRNA quantitation.

Two methods were used for the reverse transcription of the total RNA samples.

METHOD 1.

Prior to reverse transcription, total RNA samples were treated with RQ1 Rnase-free DNase I (10 U/μl) and incubated at 37^0C for 30 minutes, to degrade any contaminant DNA in the RNA samples. The reaction was set up as seen in Table **2.4.2-1**. This step was followed by the addition of RQ1-DNase stop solution, 10 min incubation at 65^0C and quick chill on ice to inactivate RQ1 Rnase-free DNase I. As a control for DNase treatment and also DNA contamination in the RNA samples, identical samples with no DNase treatment and a Nuclease free (NF) water control were included in every experiment at this point. For first strand cDNA synthesis, 2 μl of 50 μM oligo dT_{12-18} were added to 10 μl (1 μg) of the DNase treated RNA and this mixture was denatured at 70^0C for 10min and immediately chilled on ice. A mixture of first strand buffer (5X), DTT, dNTPs in excess and RNasin ribonuclease inhibitor was prepared in the meantime, under the DNA free PCR hood to avoid contamination of the reagents with DNA. One μl of Superscript II (RT samples) or 1 μl of NF H_2O for the -RT controls

(samples containing RNA which was not reverse transcribed) were added at this point to each sample. Finally, 12 µl of denatured RNA (1 µg) was added to give the final 20 µl reverse transcription mixture, see **Table 2.4.2-2**

Reactants	Amount	Final concentration
Total RNA sample	2 µg	0.1 µg/µl
First Strand Buffer (5x)	4 µl	1x
Rnase-free Dnase I (10 U/µl)	2 µl	20 units
NF H_2O	Up to 20 µl	-
Total Volume	20 µl	

Table 2.4.2-1 Removal of genomic DNA from total RNA by Rnase-free Dnase I. Samples were reverse transcribed at 42^0C for 60 min, 95^0C for 5 min and held at 4^0C in a 2200 Perkin Elmer Thermocycler.

Reactants	**Volume per PCR tube (µl)**	Final concentration
First strand Buffer (5X)	4	1x
DTT (100mM)	1	5 mM
DNTP (10mM)	1	500 µM
RNasin ribonuclease inhibitor (40 units/µl)	1	40 units
Superscript II (200 units/µl)	1	200 units
Denatured RNA mixture	12	1 µg
Total Volume	20	

Table 2.4.2-2 Protocol for the reverse transcription of RNA using Superscript II RNase H$^-$ Reverse Transcriptase.

The newly synthesized cDNA was used as a template for conventional PCR amplification as follows:

A volume of 11.2 µl from each cDNA sample was prepared for PCR reaction. The oligonucleotides used to amplify a 242 bp fragment of the HO-1 gene corresponding to nucleotides 1198 to 1440 bp region of the published heme oxygenase sequence (Yoshida *et al.*, 1988), F: 5'-CCT TGT TGA CAC GGC CAT GAC CAC-3' (P1), and R: 5'-AGT TAG ACC AAG GCC ACA GTG CCG-3' (P2). The primers were purchased from GIBCO BRL, Life Technologies, UK.

Also the expression of GAPDH gene was monitored. The two oligonucleotides used to amplify a 178 bp fragment of the GAPDH gene were F: 5'-GAC ATC AAG AAG GTG GTG AA-3' (G1) and R: 5'-TGT CAT ACC AGG AAA TGA GC-3' (G2).

For a final volume of 50 µl, the reaction mixture contained 5 µl 10XPCR buffer, 1µl dNTP (0.2 mM each), 5 µl primer P1 (0.6 µmoles), 5 µl primer P2 (0.6 µmoles), 0.5 µl of Platinum Taq Polymerase (2.5 U), 1.5 µl $MgCl_2$ (50mM) and 31 µl NF water. The reaction mix was covered with 30 µl of paraffin oil to minimise evaporation. The polymerase reaction was initiated with a denaturation and enzyme activating step at 94^0C for 5 minutes and carried on for 35 cycles with the following steps: denaturation step (94^0C for 30sec), annealing step (61^0C for 30sec), extension step (72^0C for 30sec) (last elongation step 7min). After the completion of the PCR reaction a 17 µl aliquot mixed with 3 µl of 6x Loading Buffer was electrophoresed on a 2% agarose gel to be visualised

METHOD 2

cDNA synthesis was carried out using products from Life Technologies (10x RT-buffer, dNTPs, $MgCl_2$, random hexamers, RNase H and Superscript II RNase H^- Reverse Transcriptase (RT)).

For cDNA synthesis and -RT controls (samples containing RNA which was not reverse transcribed), tubes with 3 μg RNA, 10 μl random hexamers (50 ng/μl), 2 μl dNTPs (10 mM) and DEPC-treated water added to a volume of 20 μl were heated at 65°C for 5 min and then cooled on ice for at least 1 min. An 18 μl RT-premix containing 8 μl (25mM) $MgCl_2$, 4 μl 10x RT Buffer, 4 μl DTT (100 mM) and 2 μl (80U) RNase Inhibitor was added to the RNA containing mixture (total volume 38 μl). From this mixture 19 μl were separated in a different tube for cDNA synthesis and 1 μl (50U) of Superscript II was added to each tube. The cDNA and the -RT mixtures were incubated at room temperature for 10 min, at 42°C for 50 min, at 70°C for 15 min and then cooled on ice. One μl (2U) of RNase H was added to the cDNA samples followed by an incubation at 37°C for 20 min. Finally the reactions were diluted 10 fold in DEPC treated water. Both the quality of the cDNA product and the absence of contaminant DNA in the -RT samples were tested with a conventional polymerase chain reaction (PCR) reaction that was run on a 2200 Perkin Elmer Thermocycler. The PCR amplifications were performed using primers: F: 5'-GAC CGT TCA GCT GGA TAT TAC-3' (R1) and R: 5'-GAC ATG GAA GCC ATC ACA GAC-3' (R2) and visualised on a 2% agarose gel with ethidium bromide (EtBr). This reverse transcription protocol was followed by real time PCR using the ABI PRISM 7700 Sequence Detector (Taqman) (Hoffman La Roche Laboratories, Switzerland) or the LightCycler® (Roche Molecular Systems, Mannheim, Germany) (Home Lab).

2.4.2.1 Real Time Taqman PCR

This approach utilises the inherent 5'-3' exonuclease activity of Taq DNA polymerase to cleave a specific oligonucleotide dual-labelled probe annealed to the target sequence during PCR amplification (Lie and Petropoulos, 1998; Holland *et al.*, 1991). The probe is labelled with a reporter fluorophore at the 5'end of the probe and with a quencher fluorophore at the 3' end. Cleavage of the probe during strand elongation releases the reporter from the probe and thus its proximity to the quencher, resulting in an increase in the reporter emission intensity. The cycle at which the emission intensity of the sample rises above the baseline is referred to as the theshold cycle (Ct) and is inversely proportional to the copy number of the target template. A duplex Taqman PCR was run

to assess the level of HO-1 cDNA for each sample, with use of rRNA as an internal control in each PCR reaction well. Briefly, a 74bp fragment of the HO-1 cDNA was amplified by using the primers: F: 5'-AAG AGG CCA AGA CTG CGT TC-3' (P3) and R: 5'-GGT GTC ATG GGT CAG CAG C-3' (P4). A Taqman probe, 5'FAM (6-carboxy fluorescein)-TCA ACA TCC AGC TCT TTG AGG AGT TGC AG-3' TAMRA (6-carboxy tetramethylrhodamine), which was designed to cover the exon3-exon 4 boundary in the cDNA was included in the Taqman PCR Master Mix. The rRNA primer sequences were: F: 5'-CGG CTA CCA CAT CCA AGG AA-3', R: 5'-GCT GGA ATT ACC GCG GCT-3' and the Taqman rRNA probe VIC-5'-TGC TGG CAC CAG ACT TGC CCT C-3'-TAMRA (6-carboxy tetramethylrhodamine).

2.4.2.2 Real Time PCR LightCycler® (Roche Molecular Systems, Mannheim, Germany)

Following reverse transcription, Light Cycler Real time PCR was run to assess the level of HO-1 and GAPDH cDNAs for each sample using the double-strand-specific dye SYBR Green I. After developing the methodology (described in section 3.5.2) the standard protocols for PCR amplifications were as described in this paragraph. PCR reactions of a final volume of 18 μl containing 2 μl SYBR Green I, 1.6 μl $MgCl_2$ (25 mM), 2 μl each primer (5 μM) and 10.4 μl PCR grade water were prepared under the PCR Hood. To this mixture 2 μl aliquots of reverse transcribed RNA were added. The standard protocol for PCR amplification of a 74bp fragment of the HO-cDNA by using the primers: F: 5'-AAG AGG CCA AGA CTG CGT TC-3' and R: 5'-GGT GTC ATG GGT CAG CAG C-3' consisted of an initial denaturation step at 95 ^{0}C for 30sec followed by 30 cycles of 95 ^{0}C (0sec), 52^{0}C (15sec), 72 ^{0}C (4sec), 81^{0}C (3sec). The protocol for PCR amplification of a 178bp fragment of the GAPDH cDNA by using the primers F: 5'-GAC ATC AAG AAG GTG GTG AA-3' and R: 5'-TGT CAT AAC AGG AAA TGA GC-3' consisted of a denaturation step at 95°C for 30 sec followed by 40 cycles of 95°C (0 sec), 55°C (5 sec), 72°C (7 sec). After the cycling protocol, a final step was applied to all reactions by monitoring fluorescence as a function of temperature transition at a rate of 0.2 ^{0}C/sec to generate a melting curve for each

reaction (95 ^{0}C 0sec, 55 ^{0}C 15sec, 95 ^{0}C 0sec). Serial dilutions of linearised HO-1 plasmid were used as template DNA at copy numbers ranging from $2.7x10^3$ to $2.7x10^7$ to produce the standard curve. For the quantitation of the housekeeping gene GAPDH a human cDNA control probe (Clonetech laboratories, USA) was used as template DNA at copy numbers ranging from $8.3x10^4$ to $8.3x10^7$ to produce the standard curve for the quantitation of this gene. For each sample the ratio of copy numbers was calculated and normalised against control values.

2.5 Statistical analysis

Data are given as the arithmetical mean ± SD, n=3-4. Significances were calculated using Student's t-Test. For the duplicate measurements on duplicate dishes, the data are given as the arithmetical mean of four measurements and the minimum and maximum values are represented as _ on each figure. All analyses were carried out using Origin 6.0.

3 RESULTS

3.1 Kinetics of β-carotene uptake by FEK4 cells and stability in medium under cell culture conditions.

3.1.1 Uptake of β-carotene by confluent FEK4 cells and stability in cell culture medium.

Two delivery methods were compared for their effectiveness in delivering β-carotene at 1.5 and 4.5 μM concentrations from the culture medium to confluent normal human skin fibroblasts. The first involved a 1:1 mixture of tetrahydrofuran (THF) with ethanol (EtOH) with daily medium and β-carotene solution changes, and the other used THF and a single initial addition of β-carotene to the medium of FEK4 monolayers. Neither of the solvent combinations were found to be toxic to cells at concentrations in the cell culture medium of 0.5% for THF and ≤1% for THF/EtOH (Trypan blue dye exclusion assay) (results not shown). These solvent concentrations were not exceeded in our experiments. At the selected intervals (2.5, 5, 24, 48 and 72h) the cellular content of β-carotene was evaluated by HPLC analysis (Roche Vitamins A.G., Basel) (Nierenberg *et al.*, 1989).

Briefly, after thorough washing of the cell monolayers (PBS+2% BSA, 6x5min at 37^0C) the cells were detached from their plates by trypsinization, pelleted, and the pellets frozen (-80^0C) for analysis. The stability of β-carotene in the growth medium under cell culture conditions was monitored at the same time intervals. Although THF without antioxidant is also well tolerated by FEK4 cells at this concentration, it has been reported to be occasionally cytotoxic after extended storage, possibly because of peroxide formation (Bertram *et al.*, 1991). THF containing 0.025% BHT was therefore used in all our studies. Precipitation has been reported to occur upon addition of β-carotene solutions in THF (Bertram, 1991; Offord *et al.*, 2002). No precipitation of β-carotene was observed upon addition of the β-carotene solutions to the cell culture medium with either of the tested methods, as determined with light microscopy.

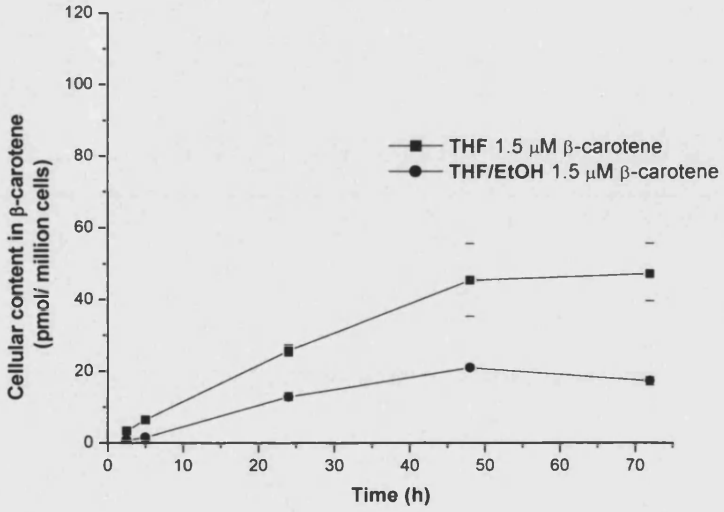


Fig. 3.1.1-1. Time dependence of cellular uptake of β-carotene. β-carotene uptake by confluent FEK4 cells when cultured for three days with 1.5 μM concentration in the growth medium, using either THF or THF/EtOH as a carrier. Results shown are the means of duplicate measurements on duplicate dishes, the minimum and maximum values represented as -.

With both the carrier systems, the uptake was time- and concentration- dependent. The delivery of β-carotene to FEK4 was optimal when THF was used as a carrier. The uptake was 2.5 or 4 fold lower when the THF/EtOH mixture was used to deliver β-carotene from 1.5 or 4.5 μM in the medium respectively **(Figs. 3.1.1-1,-2**[M K T4]). The maximum cellular β-carotene concentration achieved was 220 pmol/10^6cells, after a 3-day incubation with β-carotene dissolved in THF applied to the cell monolayers at a final medium concentration of 4.5 μM.

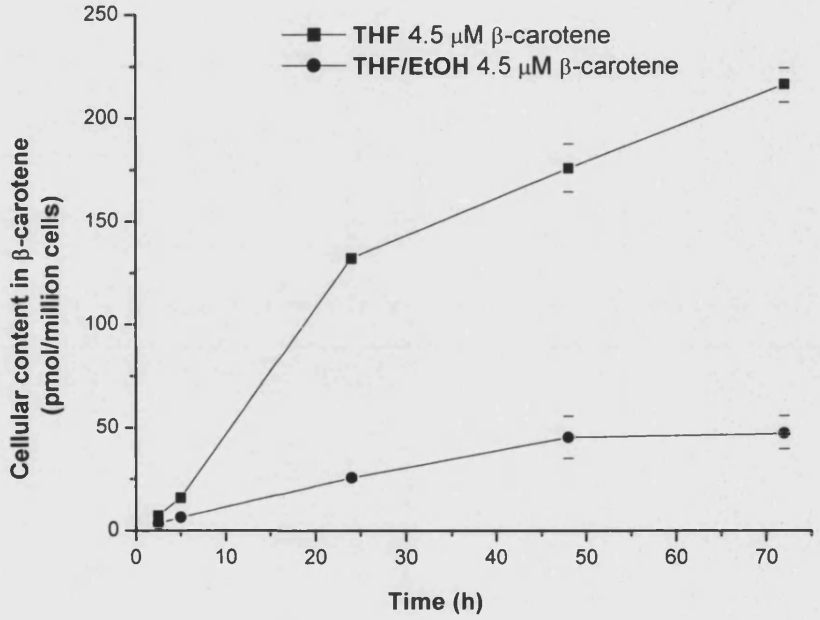


Fig. 3.1.1-2 Time dependence of cellular uptake of β-carotene. β-carotene uptake by confluent FEK4 cells when cultured for three days with 4.5 μM concentration in the growth medium, using either THF or THF/EtOH as a carrier. Results shown are the means of duplicate measurements on duplicate dishes, the minimum and maximum values represented as -.

The β-carotene concentrations in the cell culture medium were monitored over a three day period with both the delivery methods. β-carotene concentration in the medium remained constant when using THF/EtOH as a vehicle for the delivery of β-carotene to FEK4 cells. It should be noted, however, that this method included daily changes of medium and β-carotene solutions. The method using THF as vehicle for the delivery of β-carotene to FEK4 cells included a single addition of β-carotene in THF solutions and

β-carotene in the medium depleted in the 3 day observation period in a concentration dependent manner **(Figs. 3.1.1-3,-4)**. The depletion of β-carotene in three days was calculated using the formula: (Initial β-carotene medium concentration – β-carotene medium concentration 3 days later) x100/ Initial β-carotene medium concentration] and this gave a value of 3.3% when the initial concentration of β-carotene added to the medium was 1.52 μM. The amount of β-carotene taken up by confluent FEK4 cells from this concentration was 61.78 pmoles. This amount accounts for the 24.7% of the difference between the initial medium concentration and the medium concentration after 3 days (0.05 μM).

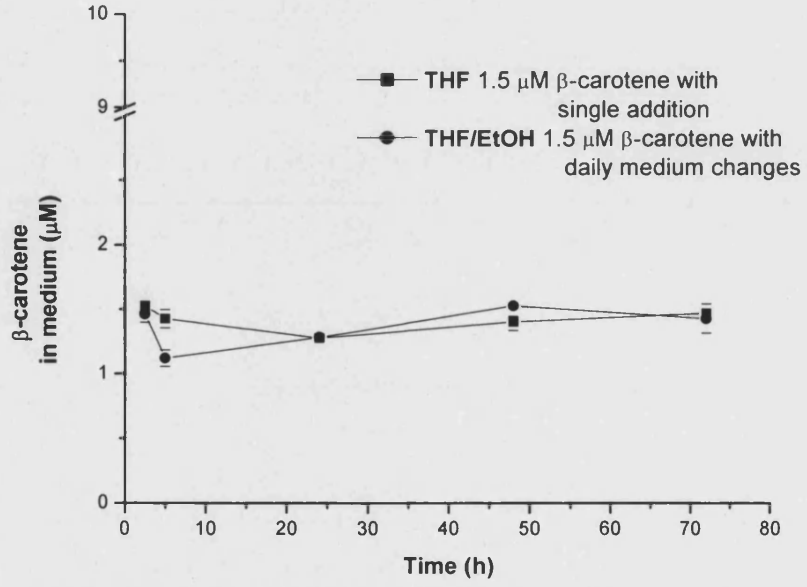


Fig. 3.1.1-3. Stability of β-carotene 1.5 μM in the medium. Stability of 1.5 μM β-carotene in the medium of confluent FEK4 cells over a three-day period, using either THF (single addition) or THF/EtOH (daily medium and β-carotene solution changes) as a carrier. Results shown are the means of duplicate measurements on duplicate dishes, the minimum and maximum values represented as -.

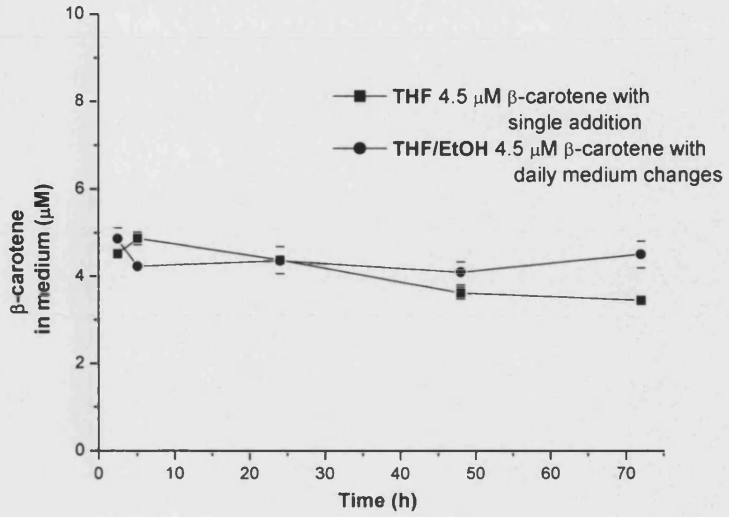


Fig. 3.1.1-4. Stability of β-carotene 4.5 μM in the medium. Stability of 4.5 μM β-carotene in the medium of confluent FEK4 cells over a three-day period, using either THF (single addition) or THF/EtOH (daily medium and β-carotene solution changes) as a carrier. Results shown are the means of duplicate measurements on duplicate dishes, the minimum and maximum values represented as -.

With THF/EtOH as a vehicle for the delivery of β-carotene to FEK4 cells no depletion was observed, because of the daily medium changes. With THF as a delivery system of β-carotene to FEK4 cells, depletion of a higher concentration (4.5 μM) of β-carotene in the medium reaches a level of 23.5% of the original concentration in three days. [M K T5]The amount of β-carotene taken up by confluent FEK4 cells by 4.5 μM in the medium (0.24 nmoles) accounts for the 4.5% of the difference between the initial β-carotene medium concentration and the medium concentration three days later.

For all the remaining following experiments we used THF as a carrier for delivering β-carotene to FEK4 cells, as higher cellular β-carotene levels would be expected to modulate the UVA induced HO-1 gene expression more effectively and the incubation time was at least 3 days (range 3-3.5).

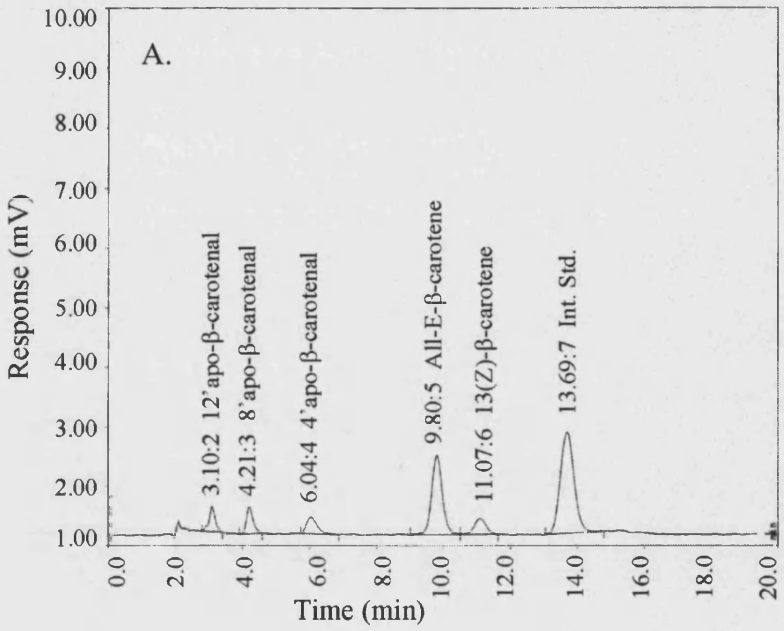
In order to weigh β-carotene and prepare the β-carotene solutions to be added to the sterile cell cultures, procedures are carried out in the glove bag under inert gas, as even traces of oxygen could lead to rapid oxidation of the compound (Britton, 1995). The

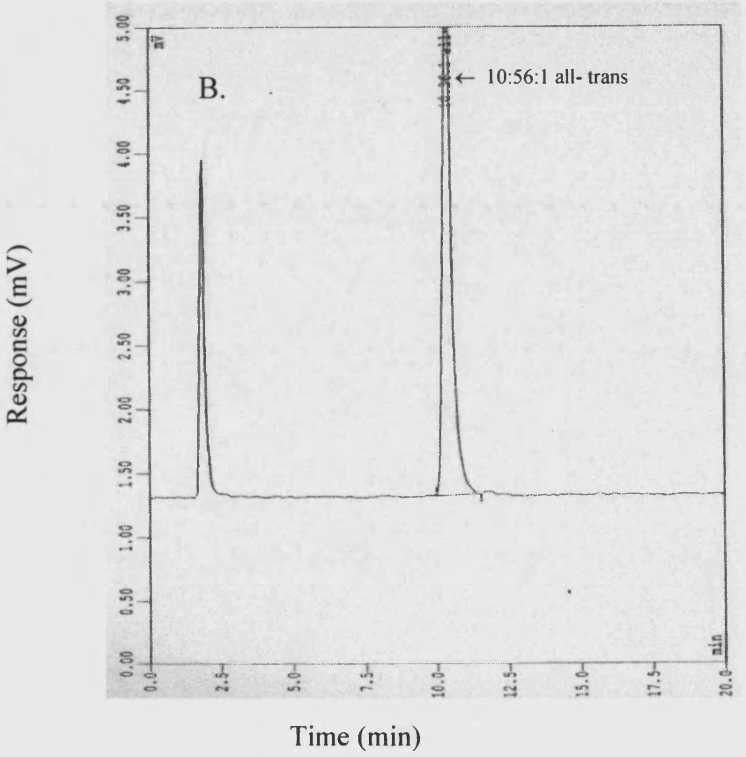
compound must also be shielded from light to avoid photo- bleaching and photo-isomerisation. Furthermore, the amounts of 99% all-trans-β-carotene are limiting. Each aliquot of crystalline β-carotene weighed out in the glove bag was approximately 10 mg. After preparing the β-carotene solutions in THF (THF, containing 0.025% BHT), the solutions were measured spectrophotometrically and the volumes to be added to the cell cultures adjusted appropriately to correct for possible weighing errors. However, using this approach BHT, present in the solvent, ranged in the culture medium from 3.8 to 5.8 μM. BHT is a well known lipophilic antioxidant and has been shown to slightly modulate UVA induced HO-1 accumulation (Kvam *et al.*, 1999; Basu-Modak *et al.*, 1996). Bearing this in mind, for all following experiments we used the solvent at a constant volume concentration in the medium (0.3%) and measured the carotenoid solutions spectrophotometrically only as a rough control for errors. The final medium concentration in β-carotene was assessed by HPLC analysis that enables an accurate measurement not only of content in carotenoids but also of isomer and metabolite formation (Nierenberg *et al.*, 1989; Stahl *et al.*, 1992; Liu *et al.*, 2000). It has been strongly suggested that investigators working with carotenoids in cell culture incorporate HPLC analysis of carotenoid stability, cellular concentrations and metabolism into their investigations in order to interpret the data obtained (Clinton, 1998). A chromatogram of standards and typical chromatograms of the separation of β-carotene and metabolites in medium samples and cell pellets are shown in (**Figs. 3.1.1-5 A, B, C**). From comparison with the standards on **Fig. 3.1.1-5A** the peak in **Fig. 3.1.1-5B** is identified as all-trans β-carotene and the peaks in **Fig. 3.1.1-5C** (from left to right) as apocarotenals, all-trans- and cis-β-carotene, respectively.

Interest in the cis-isomers of the carotenoids has been stimulated by the recognition of isomer specific biological functions that exist for the retinoids and may exist for the carotenoids (Stahl and Sies, 1993). We performed experiments designed to observe uptake of the carotenoid by FEK4 cells, stability in their growth medium, as well as isomerisation and apocarotenal formation in cells and medium samples.

β-carotene uptake from 1.1, 2.2 and 3.3 μM in the medium by FEK4 cells when THF was used at a constant volume concentration in the medium (0.3%) to deliver the carotenoid to the cells was time and concentration dependent **(Fig. 3.1.1-6)**. The

maximum cellular β-carotene concentration achieved was approximately 200 pmol/million cells. In cells β-carotene cis-isomers were present at a range between 3 and 6% of total β-carotene and apocarotenals at levels equivalent to 1.5-6% of total β-carotene **(Fig. 3.1.1-7)** whereas, no isomerisation or apocarotenal formation was detected in the medium.





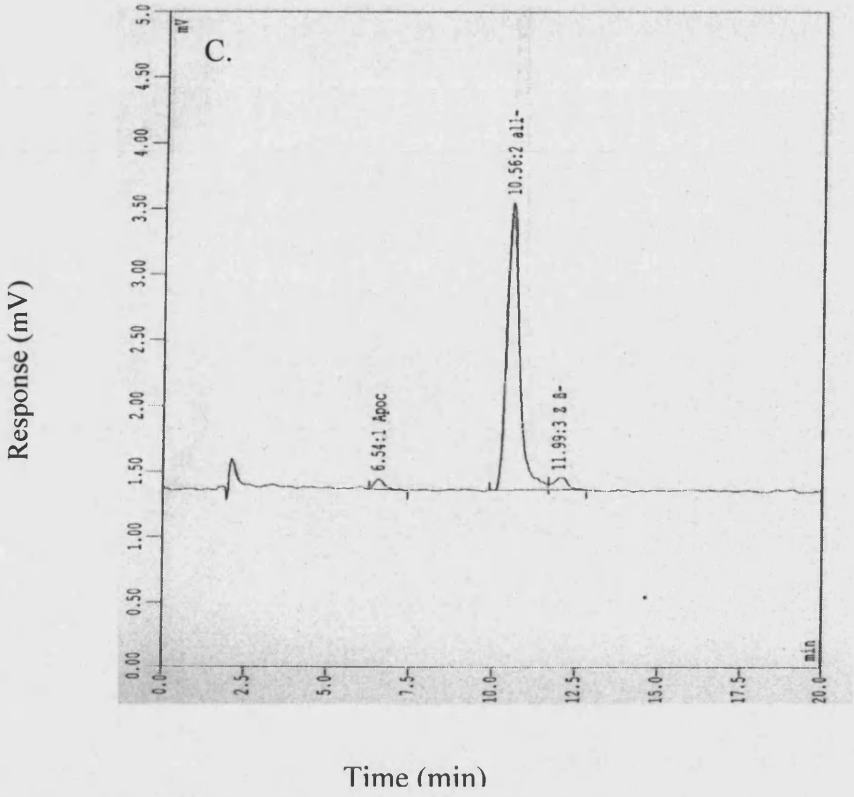


Fig. 3.1.1-5. Separation of carotenoids. (A) A mixture of 12', 8' and 4'apo-β-carotenals, all-*trans*-*β*-carotene, 13-*cis*-*β*-carotene and β-cryptoxanthin pelargonate (I.S.) in cell extracts prepared as described in Appendix 1. The compounds were detected by their absorbance at 450 nm, sensitivity: 0.01 a.u.f.s. The separation was achieved with a flow rate of 1.5 ml/min producing a back pressure of 72 bar. **(B)** A chromatogram from a medium sample and **(C)** from the corresponding cell extract. The HPLC analysis was performed in Hoffman La Roche, Basel, Switzerland by G.Riss and the cell were cultured and incubated with β-carotene in the University of Bath, Bath.

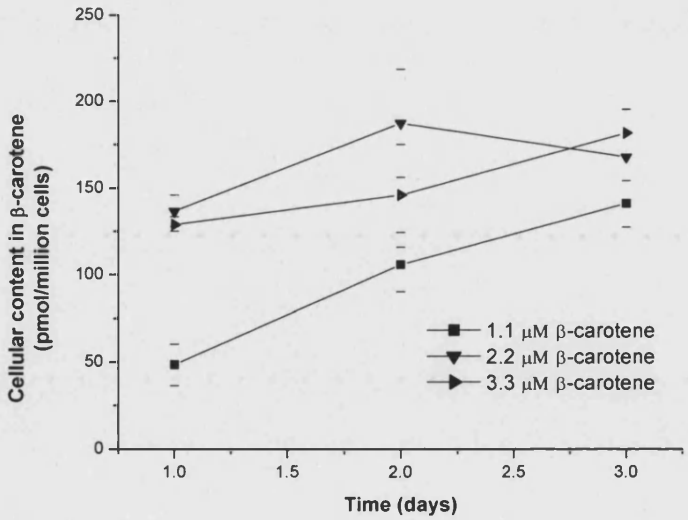


Fig. 3.1.1-6. Time and concentration dependence of 3-day cellular uptake of β-carotene by confluent FEK4 cells. Beta-carotene uptake by confluent FEK4 over a 3-day period, when cultured with 3 different β-carotene concentrations (1.1, 2.2 and 3.3 μM) in the medium, using THF as a carrier. Results shown are the means of duplicate measurements on duplicate dishes, the minimum and maximum values represented as -.

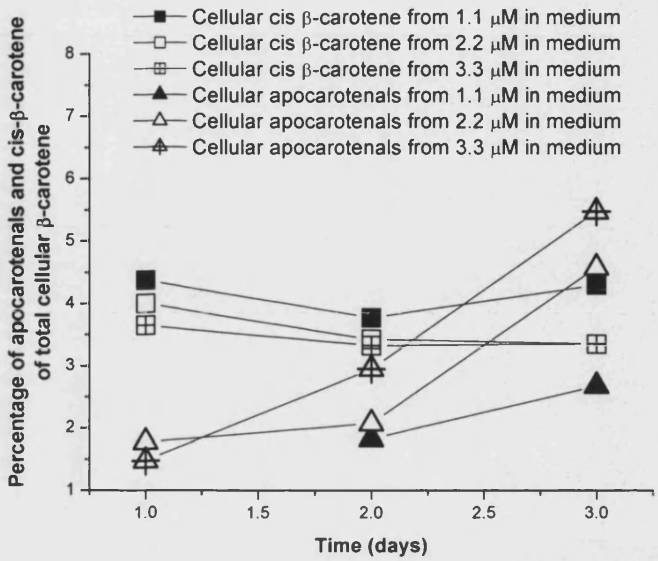


Fig. 3.1.1-7. Percentage of cis-β-carotene and apocarotenals of total cellular β-carotene in confluent FEK4 cells. Percentage of cis-β-carotene and apocarotenals of total cellular β-carotene from 1.1, 2.2 and 3.3 μM in the medium over a 3-day period, using THF as a carrier. Results shown are the means of duplicate measurements on duplicate dishes, the minimum and maximum values represented as -.

As in the previous set of experiments, β-carotene in the medium depleted in a concentration dependent manner **(Fig. 3.1.1-8)**. The maximum percentage of depletion of 1.1, 2.2 and 3.3 μM β-carotene was the depletion of 3.3 μM β-carotene in the medium and was approximately 20% **(Fig. 3.1.1-9)**. The quantity of β-carotene taken up by confluent FEK4 cells at this concentration (3.3 μM) accounted for only the 5% of the observed loss from the medium.

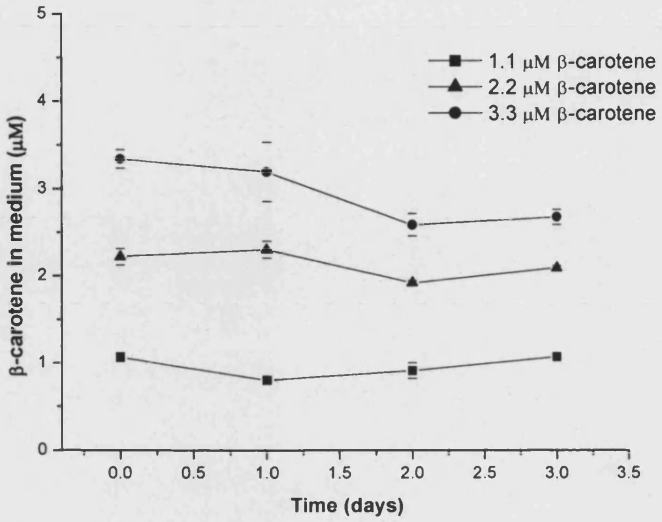


Fig. 3.1.1-8. Stability of 1.1, 2.2 and 3.3 μM β-carotene in the medium of confluent FEK4 cells. Stability of 1.1, 2.2 and 3.3 μM β-carotene in the medium of confluent FEK4 cells over a three-day period, using THF as a carrier. Results shown are the means of two measurements. The minimum and maximum values represented as -. The signs for minimum and maximum values are not shown when they are almost identical to the means.

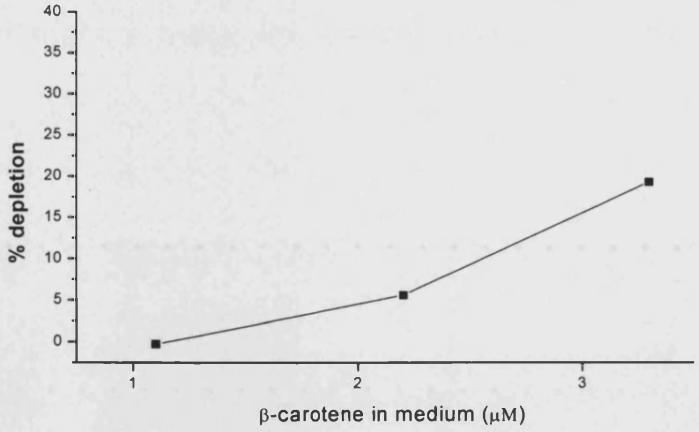


Fig. 3.1.1-9. Concentration dependence of β-carotene depletion. Percentage of depletion of initial β-carotene concentration in three days relative to initial concentration. [%Depletion= (Initial β-carotene concentration -β-carotene concentration after 3 days/ Initial -β-carotene concentration) x100].

3.1.2 Uptake of β-carotene by exponentially growing FEK4 cells and stability in the cell culture medium.

In order to monitor the uptake of β-carotene by FEK4 cells under the optimum conditions for measuring UVA induced HO-1 mRNA accumulation in cultured fibroblasts and to optimise our experimental conditions accordingly, we performed experiments to monitor uptake of β-carotene by exponentially growing FEK4 cells.

We introduced β-carotene to the cell cultures at the time of initial seeding and adjusted the seeding density so that the fibroblasts would be in exponentially growing phase and at numbers that would give a sufficient RNA yield for Northern Blot analysis 3.5 days later ($3.5x10^5$ cells/10cm dish), the day of UVA irradiation of FEK4 cultures. To enable monitoring of cellular β-carotene levels under these conditions, a range of β-carotene concentrations (0.08, 0.3, 0.9, 2.5, 9.0 and 23.0 μM) was added to the growth medium of exponentially growing FEK4 cells at initial seeding and the cells were incubated for three days, point at which cells were detached from their plates, pelleted and frozen to be sent for HPLC analysis (See Methods).

Cellular β-carotene concentration increased with increasing levels of β-carotene in the medium at the lower concentrations tested (0.08, 0.3, 0.9, 2.5 μM) but reached a plateau at medium concentrations greater than 2.5 μM **(Fig. 3.1.2-1)**. The maximum cellular β-carotene concentration achieved was ~200 pmol/10^6cells (equivalent to ~8nmol/g wet tissue (IARC Working Group for the Evaluation of Cancer, 1998).

Isomerization of all-trans-β-carotene to its cis- isomers (9- and 13-cis-β-carotene) occurred in cells and the maximum isomerization level observed was 17%.

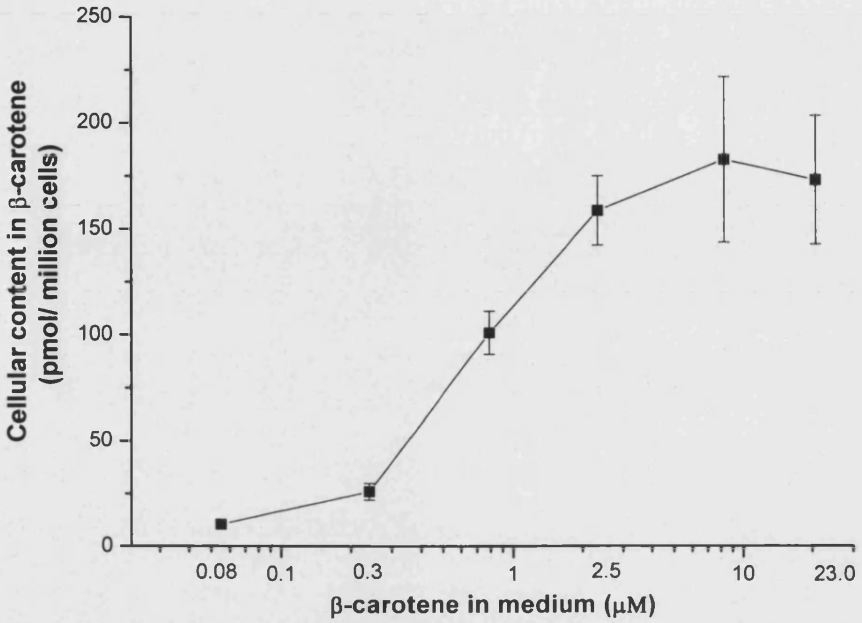


Fig. 3.1.2-1. Concentration dependence of 3 day cellular uptake of β-carotene by exponentially growing FEK4 cells. Uptake of β-carotene by FEK4 when cultured for 3 days with 6 different β-carotene concentrations (0.08, 0.3, 0.9, 2.5, 9 and 23 μM), in the growth medium. Results are means +/- SE, n=5-8.

The extent of isomerization decreased as the medium concentrations applied to the cultures and the cellular levels of β-carotene increased **(Fig. 3.1.2-2)**. The formation of apocarotenals in FEK4 followed a different pattern and detectable metabolism or degradation of cellular β-carotene to apocarotenals occurred only at the higher cellular β-carotene levels. The extent of apocarotenal formation in cells appears to be dependent on the cellular FEK4 β-carotene content and on the β-carotene medium concentration. It was different between the concentrations that resulted in a cellular β-carotene concentration of ~200 pmol/million cells reaching a maximum of 18% conversion of total cellular β-carotene to apocarotenals in cells incubated with 23 μM β-carotene for 3 days and with cellular β-carotene level ~200 pmol/ million cells **(Fig. 3.1.2-2)**.

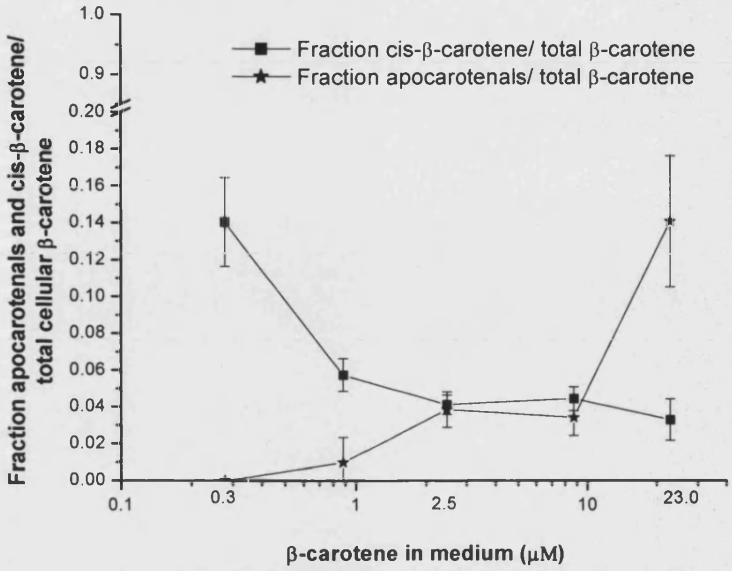


Fig. 3.1.2-2. Concentration dependence of fraction apocarotenals and cis–β-carotene of total cellular β-carotene. Apocarotenal formation and isomerisation of cellular β-carotene in FEK4 cultured for 3 days with 5 different β-carotene concentrations (0.3, 0.9, 2.5, 9 and 23 μM), in the growth medium. Results are means +/- SD, n=4-6.

In preliminary experiments performed in Hoffman La Roche laboratories by A. Wyss to measure mRNA expression of β',β-carotene-15, 15' monooxygenase (central cleavage enzyme) and of 9'-10' dioxygenase (excentric cleavage enzyme) in samples from FEK4 cells it was shown that the only enzyme present at the mRNA level was 9'-10' dioxygenase, which catalyses the cleavage of β-carotene to apocarotenals.

Under the conditions described above for monitoring of uptake of β-carotene by exponentially growing FEK4 cells, β-carotene added to the medium degraded during the 3-day incubation period and the effect increased as a function of increasing carotenoid concentrations (0.08, 0.3, 0.9, 2.5, 8.7 and 23 μM). When calculated as (Initial β-carotene medium concentration – β-carotene medium concentration 3 days later) x100/ Initial β-carotene medium concentration) it is clear that the depletion reaches a level of 90% for the highest concentration used (23 μM) **(Fig. 3.1.2-3)**. The amount of β-carotene taken up by exponentially growing FEK4 cells cannot account for the depletion from the medium, as it corresponds to 0.09-11.23% of the observed loss from the medium.

Similar to the observations made in the medium of confluent FEK4 monolayers, no isomerization of all-trans-β-carotene to its cis-isomer occurred in the medium under cell culture conditions during the three-day period, whereas in cells, cis-isomers were detected.

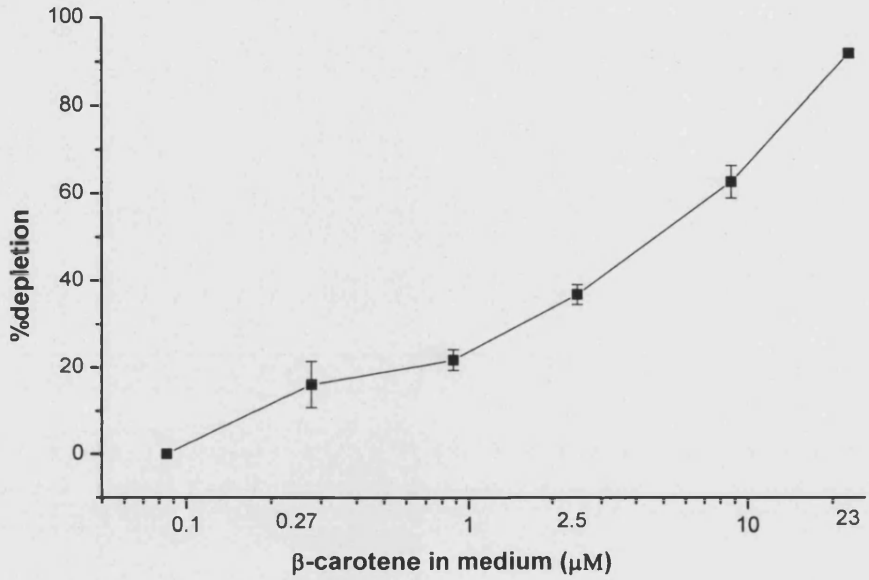


Fig. 3.1.2-3. Concentration dependence of β-carotene depletion. Percentage of depletion of initial β-carotene concentration in three days relative to initial concentration. [%Depletion= (Initial β-carotene concentration -β-carotene concentration after 3 days/ Initial -β-carotene concentration) x100]. Results are means +/- SE, n=8.

Uptake by exponentially growing FEK4 cells from 1, 2.7, 3.3 μM β-carotene in the medium seeded at a density of $3x10^5$ cells per 10cm dish reached a level of approximately 300 pmol/million cells in three days (**Fig. 3.1.2-4).** This cellular concentration of β-carotene in FEK4 cells exceeds that observed when the cells were seeded at higher a density of $3.5x10^5$ cells per 10 cm dish and incubated for three days with 0.9 and 2.5 μM β-carotene in the medium (~110 and 180 pmol/million cells,

respectively) **(Fig. 3.1.2-1)**. A similar finding of increased cellular uptake when cells are seeded at low densities has been previously described (Iftikhar *et al.*, 1996).

Fractions of cis-β-carotene and apocarotenals occurred in cells at levels 0.02-0.03 and 0.04-0.07 of total β-carotene, respectively.

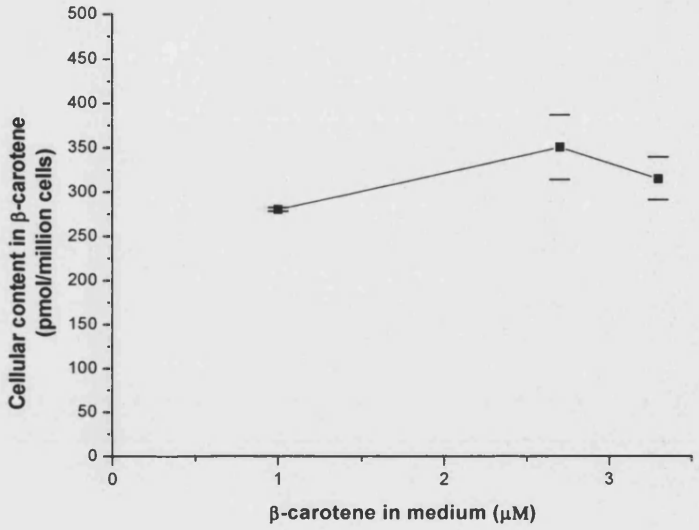


Fig. 3.1.2-4. Uptake of β-carotene by exponentially growing FEK4 cells from 1, 2.7 and 3.3 μM in the growth medium. Uptake of β-carotene by FEK4 when cultured for 3 days with 3 different β-carotene concentrations (1, 2.7 and 3.3 μM), in the growth medium. Results shown are means, of duplicate measurements on duplicate dishes minimum and maximum values shown as -.

β-carotene at concentrations of 2.7 and 3.3 μM in the growth medium of exponentially growing FEK4 cells depleted, and the depletion of β-carotene in the medium of cells increased with increasing concentrations. The maximum percent depletion observed was ~40% at the highest concentration used (3.3 μM) **(Fig 3.1.2-5)**. Consistent with our previous observations no detectable isomerization or apocarotenal formation occurred in the medium.

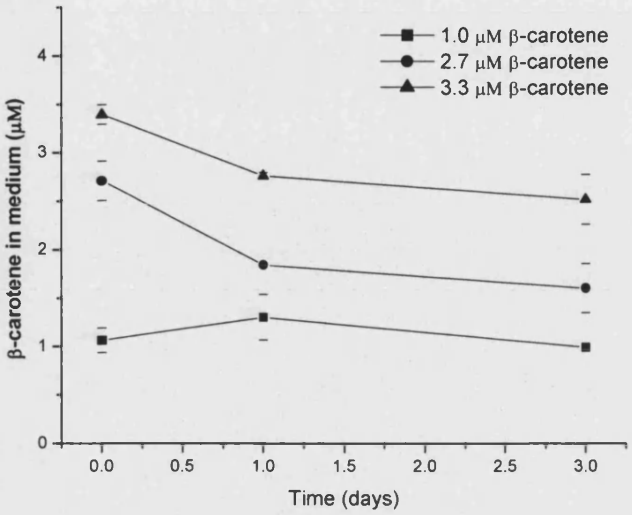


Fig. 3.1.2-5. Stability of β-carotene in the medium at concentrations of 1.0, 2.7 and 3.3 μM. Stability of β-carotene at concentrations 1.0, 2.7 and 3.3 μM in the medium of exponentially growing FEK4 cells over a three day period, using THF as a carrier. Results are the means of two measurements on medium samples from two dishes; the minimum and maximum values represented as -. The signs for minimum and maximum values are not shown when they are almost identical to the means.

3.1.3 Uptake of β-carotene by growth arrested FEK4 cells and stability in the cell culture medium.

The uptake of β-carotene by growth arrested FEK4 cells was monitored under conditions as close as possible to the conditions we used for monitoring uptake by FEK4 cells in exponentially growing phase (see Materials and Methods). When arrested cell populations (pre-equilibrated for 7 days in 0.5% serum medium) were incubated with β-carotene at concentrations 1, 1.8 and 2.5 μM in the medium for 3 days, the maximum cellular content in β-carotene was ~60 pmol/million cells **(Fig. 3.1.3-1).** There was no evidence of a concentration dependence of the uptake in the range between 1 and 2.5 μM. When compared to the uptake of β-carotene by FEK4 in other growth phases the cellular content in β-carotene in growth arrested cells was lower with uptake being 2-4 fold lower from medium concentrations in the range between 1 and 2.5

μM. Isomerization and apocarotenal formation from all-trans-β-carotene occurred in growth arrested cells and fractions of the sum of 9- and 13-cis-β-carotene as well as fractions of apocarotenals were between 0.03 and 0.05.

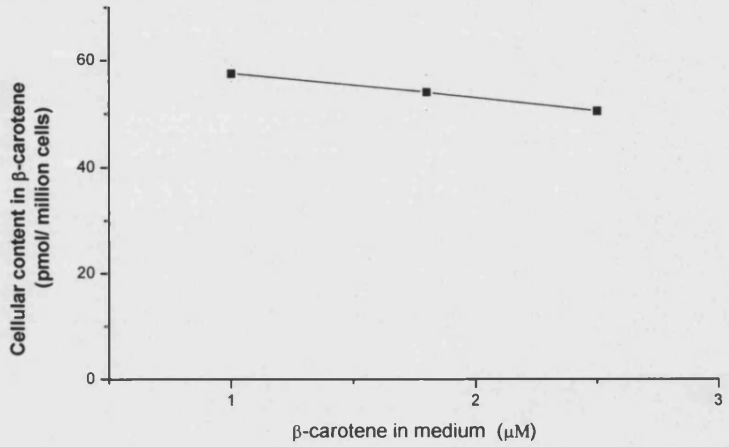


Fig. 3.1.3-1 Uptake of β-carotene by growth arrested FEK4 cells. β-carotene uptake by growth arrested FEK4 cells in three days by 1, 1.8 and 2.5 μM in the medium. Results are the means of duplicate measurements on duplicate dishes, the minimum and maximum values represented as -. The signs for minimum and maximum values are not shown when they are almost identical to the means.

During the three day observation β-carotene depleted in the medium of growth arrested FEK4 cells **(Fig. 3.1.3.-2a).** In this case as well, no isomerization of all-trans-β-carotene nor apocarotenal formation was detectable in the growth medium. Degradation of β-carotene in the medium reached a maximum of ~30% depletion of the initial concentration in three days **(Fig. 3.1.3-2b)**. The amount of β-carotene lost from the medium was not due to uptake by arrested cells, which accounted for less than 5%.

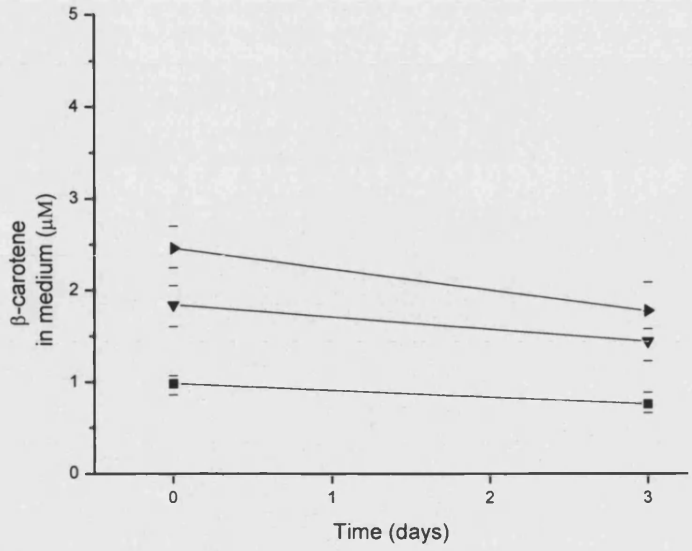


Fig. 3.1.3-2.a. Stability of β-carotene in the medium of growth arrested FEK4 cells. Stability of 1, 1.8 and 2.5 μM β-carotene in the medium of growth arrested FEK4 cells over a three-day period. Results are means of duplicate measurements on duplicate dishes, the minimum and maximum values represented as -.

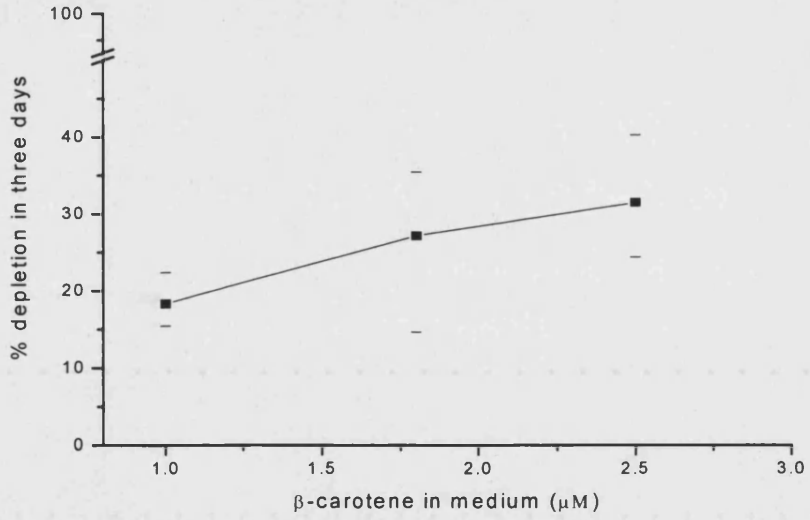


Fig. 3.1.3-2b. Concentration dependence of β-carotene depletion. Percentage of depletion of initial β-carotene concentration in three days relative to initial concentration. [%Depletion= (Initial β-carotene concentration – β-carotene concentration after 3 days /Initial–β-carotene concentration) x100]. Results are means of duplicate measurements on duplicate dishes, the minimum and maximum values represented as -.

Overall, the uptake of β-carotene by FEK4 appears to be dependent on their growth phase. Exponentially growing FEK4 at low confluency took up β-carotene more readily than FEK4 in the other growth phases. The maximum cellular level of β-carotene achieved in the three day period from approximately 1, 2, and 3 μM concentrations of the carotenoid in the medium was ~300 pmol/10^6cells in exponentially growing cells (when seeded at a density of $3x10^5$ cells per 10 cm dish), ~180 pmol/10^6cells for the confluent FEK4 monolayers and ~60 pmol/10^6cells in growth arrested FEK4 cells. Detectable isomerization of all-trans-β-carotene to its cis- isomer in the cells and metabolism to apocarotenals was observed at all growth phases and at similar levels. Cis formation ranged from 2.5 to 5% of total cellular β-carotene and apocarotenal formation ranged from 2.5 to 6% of total cellular β-carotene. Overall, the stability of similar β-carotene concentrations (approximately 1, 2, 3 μM) in the medium of FEK4 cells in different growth phases is greater in the growth medium of confluent FEK4 monolayers (maximum percentage of depletion ~20%) than in the culture medium of growth arrested (maximum percentage of depletion ~30%) or exponentially growing FEK4 (maximum percentage of depletion ~45%).

3.1.4 Spectrophotometry of β-carotene in THF

The β-carotene solutions to be added to the cell cultures were measured spectrophotometrically to control for errors in the weighing procedure to prepare solutions. To our knowledge the extinction coefficient of β-carotene in tetrahydrofuran (THF) is not published and we did the appropriate experiments to define it.

After performing wavelength scans of β-carotene solutions in THF the maximum absorption for β-carotene in this solvent was determined to be 459nm. At that wavelength we measured the absorption of β-carotene at concentrations ranging from 1 to 5 μM and calculated the extinction coefficient ϵ=135,110 cm^2/mol, λ_{max} 459.

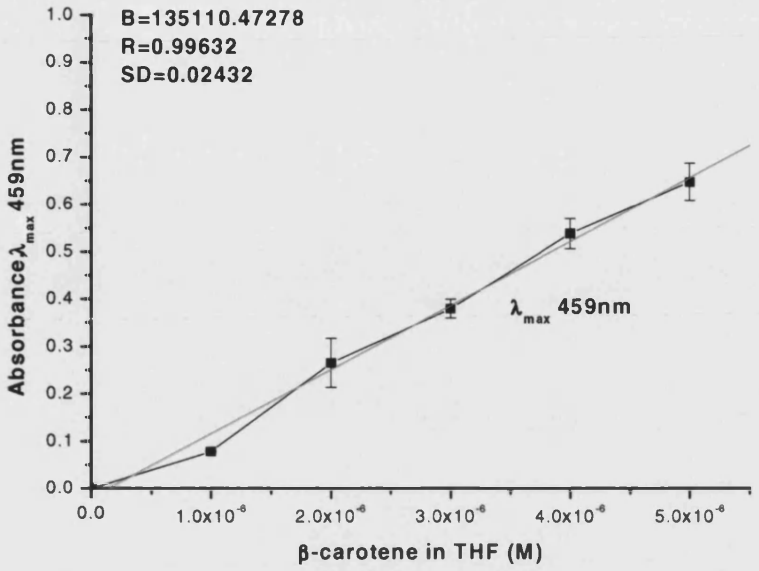


Fig.3.1.4-1 Standard curve for spectrophotometry of β– carotene in THF, λmax 459nm. Results are means +/- SD, n=3

3.2 Degradation of cellular β-carotene by UVA irradiation of FEK4 fibroblasts

Degradation of cellular β-carotene content by 250 kJ/m^2 of UVA in FEK4 fibroblasts was considerable in all growth phases (**Fig. 3.2-1,-2,-3).** Interestingly, although the percentage of degradation of cellular β-carotene was similar for both growth arrested and exponentially growing FEK4 cells being approximately 70%, cellular β-carotene appears to degrade less in cells forming confluent FEK4 monolayers with a 38% depletion observed **(Fig. 3.2-4).**

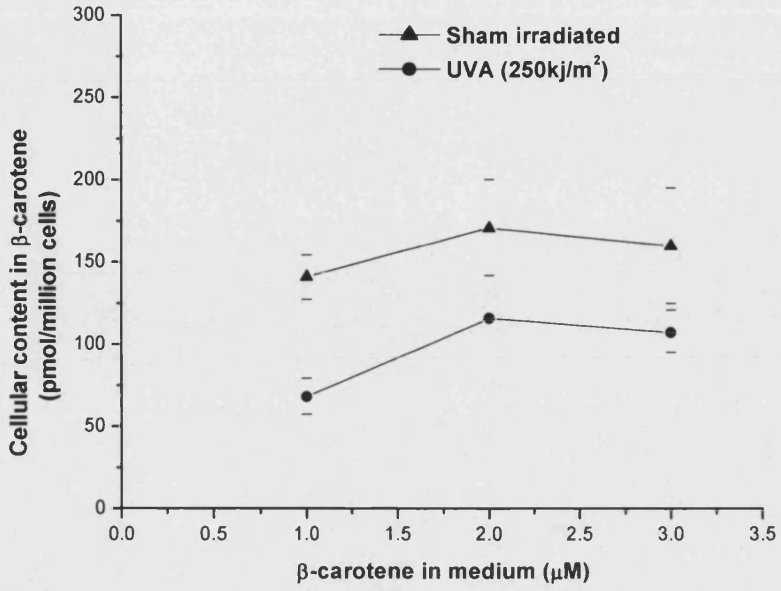


Fig. 3.2-1. Degradation of cellular β-carotene content by UVA 250 kJ/m^2 in confluent monolayers. Degradation of cellular β-carotene content by UVA 250 kJ/m^2 in confluent FEK4 monolayers Results shown are the means of duplicate measurements on duplicate dishes, the maximum and minimum values represented as -.

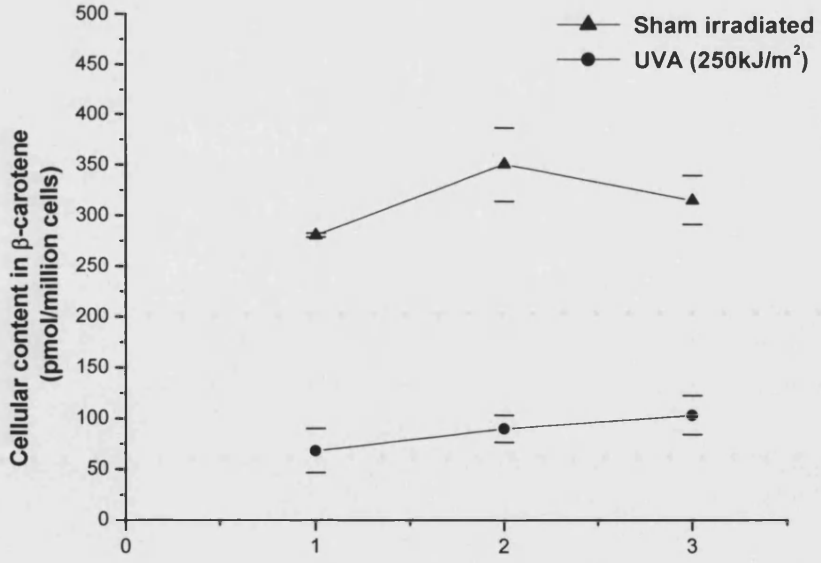


Fig. 3.2-2. Degradation of cellular β-carotene content by UVA 250 kJ/m^2 in exponentially growing FEK4 cells. Results shown are the means of duplicate measurements on duplicate dishes, the maximum and minimum values represented as -.

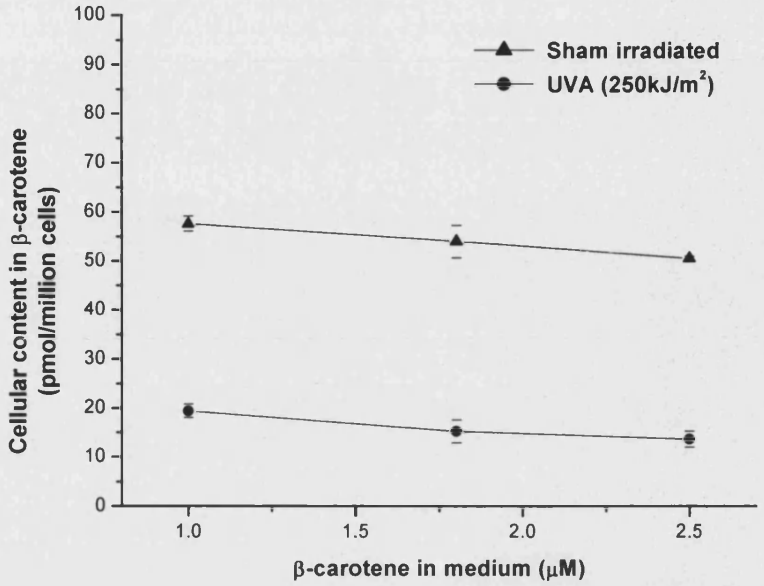


Fig. 3.2-3 Degradation of cellular β-carotene content by UVA 250 kJ/m² in growth arrested FEK4 monolayers. Results shown are means of duplicate measurements on duplicate dishes, the maximum and minimum values represented as -.

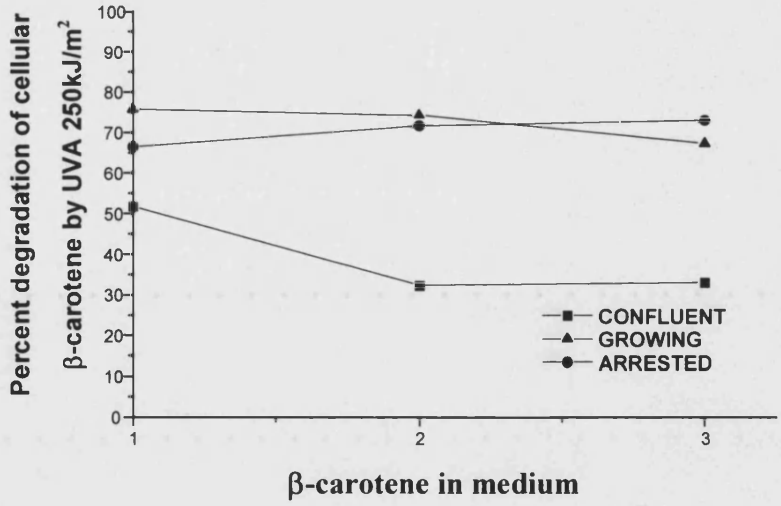


Fig. 3.2-4 Degradation of cellular β-carotene by UVA (250 kJ/m²). The percentage of degradation of cellular β-carotene by UVA (250 kJ/m²) in confluent, exponentially growing and growth arrested FEK4 monolayers. Data are derived from Figs **3.2-1,-2,-3**.

Furthermore, in all three cases UVA irradiation (250 kJ/m^2) resulted in a significant ($p<0.05$) increase in cellular levels of apocarotenals (Data shown for growing cells only, **(Fig.3.2.-5).**

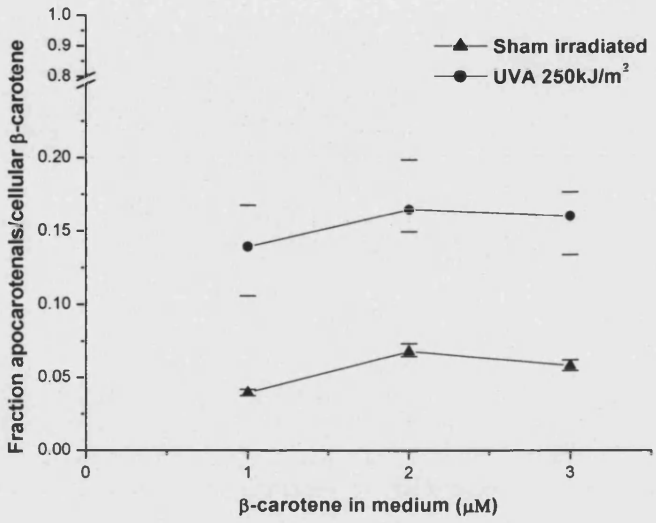


Fig. 3.2-5. The level of apocarotenals as a fraction of total cellular β-carotene content following UVA irradiation (250 kJ/m^2). Controls are identical sham irradiated FEK4 monolayers. Results are means of duplicate measurements on duplicate dishes, the minimum and maximum values represented as -.

3.3 Kinetics of lycopene uptake by FEK4 cells and stability in the medium

3.3.1 Uptake of lycopene by exponentially growing FEK4 cells and its stability in the medium.

The uptake of 96.7% all-trans lycopene by exponentially growing FEK4 was monitored over a 4-day period. The maximum cellular concentration achieved in the three day period being ~75 pmol lycopene /million cells from 6.7 μM in the medium for 2 days **(Fig. 3.3.1-1)**.

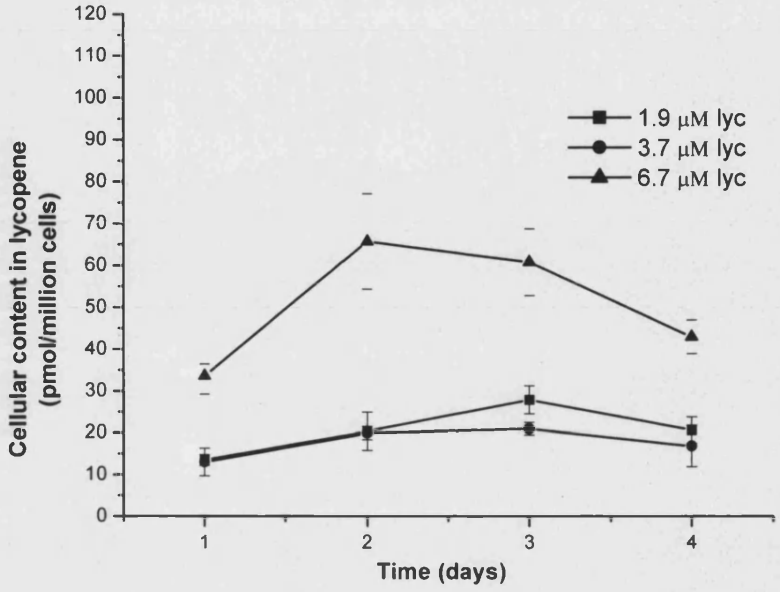


Fig. 3.3.1-1. Lycopene uptake by exponentially growing FEK4 cells over 4 days from 1.9, 3.7, and 6.7 μM lycopene in the medium. Results shown are the means, +/- SD (n=3), or the means and minimum and maximum values of duplicate measurements on duplicate dishes, the minimum and maximum values represented as -.

In 3 days the maximum cellular content in lycopene was ~65 pmol lycopene/million cells and was further reduced after the third day of incubation. When exponentially growing FEK4 cells were incubated for three days with lycopene at concentrations of 1.9 or 3.7 μM in the medium, the maximum cellular level observed was ~31.7 and ~22 pmol/million cells, respectively. When compared to the level of cellular β-carotene concentration of ~150 pmol/million cells or ~180 pmol/million cells achieved after a 3 day incubation of exponentially growing FEK4 cells with 2.5 μM or 9 μM β-carotene in the medium (see **Fig. 3.1.2-1** for comparison) the uptake of lycopene by exponentially growing FEK4 cells appears to be 3 to 5 fold lower.

In cells the isomers detected were all-trans and 5-cis lycopene, the major isomer being the all-trans that accounted for 82-100% of total lycopene. The 5-cis isomer not detectable on the first day of incubation, accounted for the 5-20% of total lycopene on the second day of incubation, and for 5-7% and 3-5% of total lycopene on the third and fourth days of incubation, respectively **(Fig. 3.3.1-2)**.

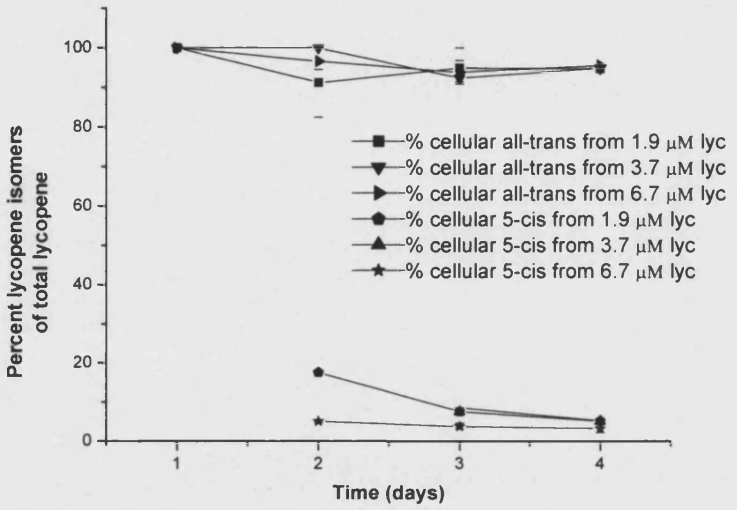


Fig. 3.3.1-2. Cellular lycopene isomers when cells were incubated with 1.9, 3.7, and 6.7 μM of the carotenoid in the cell culture medium for 4 days. Results shown are means, minimum and maximum values of measurements on duplicate or triplicate dishes, the minimum and maximum values represented as -. The signs for minimum and maximum values are not shown when they are almost identical to the means.

The percentage of the 5-cis lycopene isomer of total lycopene in cells was inversely related to the concentration of lycopene in the medium of exponentially growing FEK4 cells. When the cells were incubated with a concentration of 1.9 μM lycopene in the medium the percentage accounted for ~ 20% of total cellular lycopene whereas this percentage was ~5% when the concentration of lycopene in the medium was 6.7 μM.

The stability of lycopene in the growth medium of exponentially growing FEK4 monolayers was monitored over a four-day period for three lycopene concentrations (1.9, 3.7 and 6.7 μM) in the medium **(Fig. 3.3.1-3)**. Total lycopene in the medium depleted rapidly and depletion increased with increasing medium concentrations. After

a 1-day incubation the % depletion [% depletion = (Initial lycopene concentration - lycopene concentration after 1 day/ Initial lycopene concentration) x 100] was 40, 50 and 59% of initial lycopene medium concentrations of 1.9, 3.7 6.7 μM, respectively. The maximum % depletion observed was 75% for the highest concentration tested (6.7 μM) after a 4-day incubation period.

Three isomers were detected in the growth medium of FEK4 cells all-trans-, 5-cis and 13-cis-lycopene. On day 1 of lycopene addition to the cell cultures the all-trans lycopene and accounted for the 97% of total lycopene in the medium. In all tested concentrations of lycopene in the medium this was the major isomer detected and accounted for 86-100% of total lycopene in the medium **(Fig. 3.3.1-4a)**. The maximum level of 5-cis-lycopene detected was 8% and that of 13-cis-lycopene 6%, when expressed as percentage of total lycopene in the medium **(Fig. 3.3.1-4b and -4c)**.

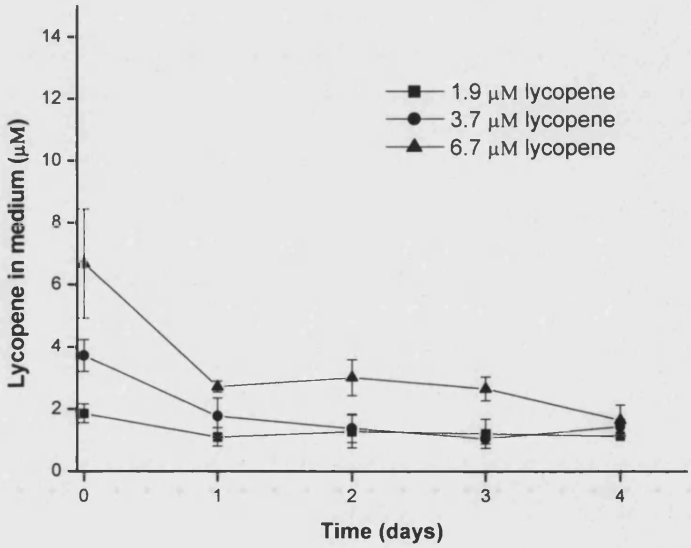


Fig. 3.3.1-3. Stability of 1.9, 3.7 and 6.7 μM lycopene in the medium of exponentially growing FEK4 cells. Stability of 1.9, 3.7 and 6.7 μM lycopene in the medium of exponentially growing FEK4 cells over a four-day period, using THF as a carrier. Results are means +/- SD, n=3.

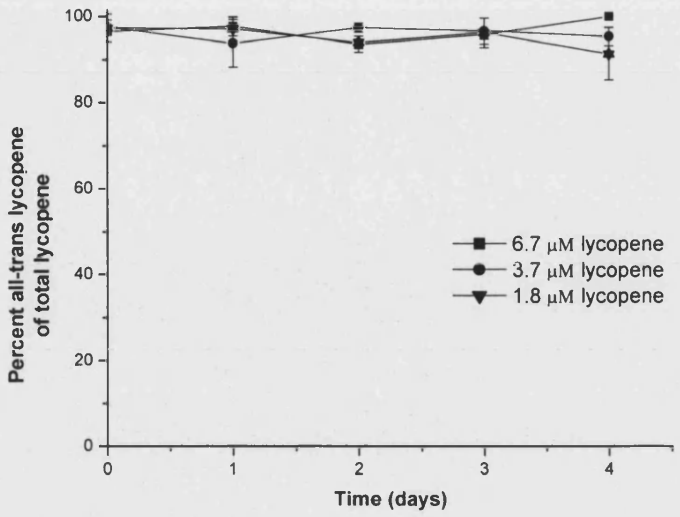


Fig. 3.3.1-4a. Percentage of all-trans-lycopene of total lycopene in the growth medium of exponentially growing FEK4 cells over a 4-day period. Percentage of all trans lycopene from 1.9, 3.7 and 6.7 μM initial lycopene concentrations applied to the cell cultures monitored over a four day period. Results are means +/- SD, n=3.

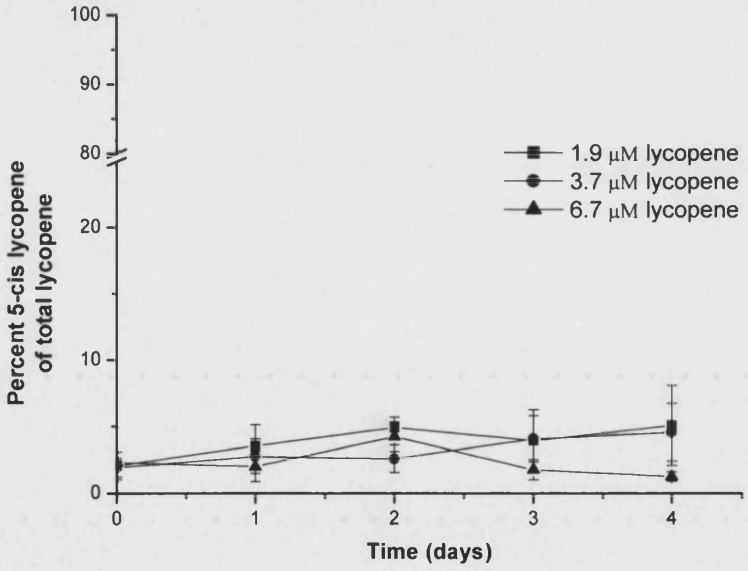


Fig. 3.3.1-4b. Percentage of 5-cis-lycopene of total lycopene in the growth medium of exponentially **growing FEK4 cells.** Percentage of 5-cis lycopene from 1.9, 3.7 and 6.7 μM initial lycopene concentrations applied to the cell cultures over a 4-day period. Results are means +/- SD, n=3.

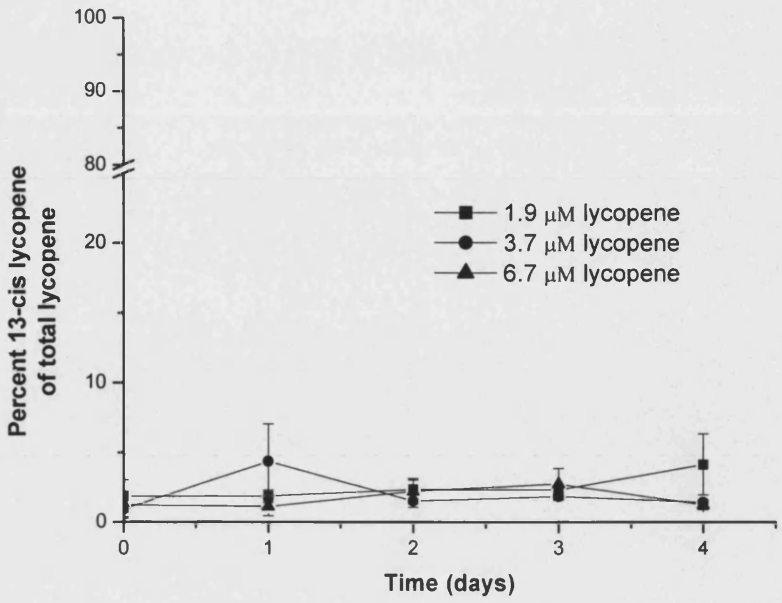


Fig. 3.3.1-4c. Percentage of 13-cis-lycopene of total lycopene in the growth medium of exponentially growing FEK4 cells Percentage of 13-cis-lycopene from 1.9, 3.7 and 6.7 μM initial lycopene concentrations applied to the cell cultures monitored over a four day period. Results are means +/- SD, n=3.

Overall, the stability of lycopene, as well as that of β-carotene, in the cell culture medium was poor over the three- to four-day observation period. To our knowledge this finding is in agreement with all publications regarding delivery of carotenoids in THF. We observed a concentration dependent depletion of the carotenoids in the medium. A similar finding has been reported by Bertram (1991), who suggested that the relative stability of the lower carotenoid concentrations could be attributed to the binding of β-carotene to protein components in the medium. Superior stability of carotenoids in the medium has been described when carotenoids were introduced to the cell cultures as β-carotene enriched bovine serum or in methyl-β-cyclodextrin, nanoparticle and in beadlet formulations, but different problems are associated with each delivery system See introduction). Precipitation of carotenoids upon addition to the culture medium has been occasionally reported (Lowe *et al.*, 1999; Offord *et al.*, 2002; Bertram *et al.*, 1991). No precipitation was observed in the experiments presented here, as seen macroscopically or by use of light microscopy. Furthermore, the isomer composition of the medium samples on Day0 was identical to that of the carotenoid in the crystalline state,

indicating that no isomerisation occurred during the preparation of the carotenoid solutions in THF.

3.3.2 Spectrophotometry of lycopene in THF

The lycopene solutions to be added to the cell cultures were measured spectrophotometrically to control for errors in the prepared solutions. To define the extinction coefficient of lycopene in tetrahydrofuran (THF), we performed wavelength scans of lycopene solutions in THF and the maximum absorption for lycopene in this solvent was defined to be 480nm. At that wavelength we measured the absorption of lycopene at concentrations ranging from 1 to 5μM and calculated the extinction coefficient ϵ=21262 cm^2/mol, λ_{max} 480.

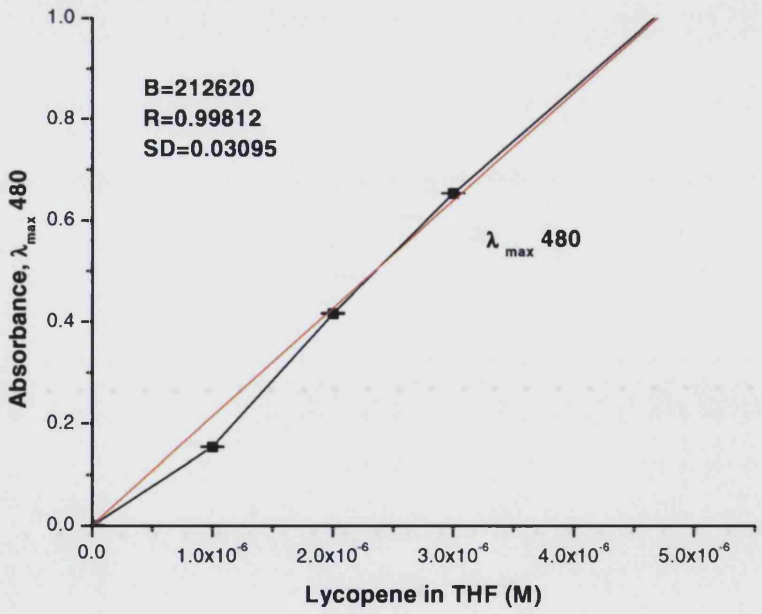


Fig. 3.3.2-1. Standard curve for spectrophotometry of lycopene in THF, λmax 480nm. Results are the means +/- SD, n=3. Error bars are smaller than the symbol when not shown.

3.4 Growth Curves

When β-carotene was added at initial seeding of FEK4 cells at concentrations 0.5, 5 and 6 μM it did not significantly affect growth of FEK4 cells in three days, when compared to control cells **(Fig. 3.4-1)**. These results are in agreement with published results regarding the effect of β- carotene on growth of normal human cells (Frommel *et al.*, 1995; Okai and Okai, 1996). β-carotene reduces growth of tumor cell lines (Palozza *et al.*, 2001; Iftikhar *et al.*, 1996; Karas *et al.*, 2000; Nara *et al.*, 2001; Prakash *et al.*, 2001).

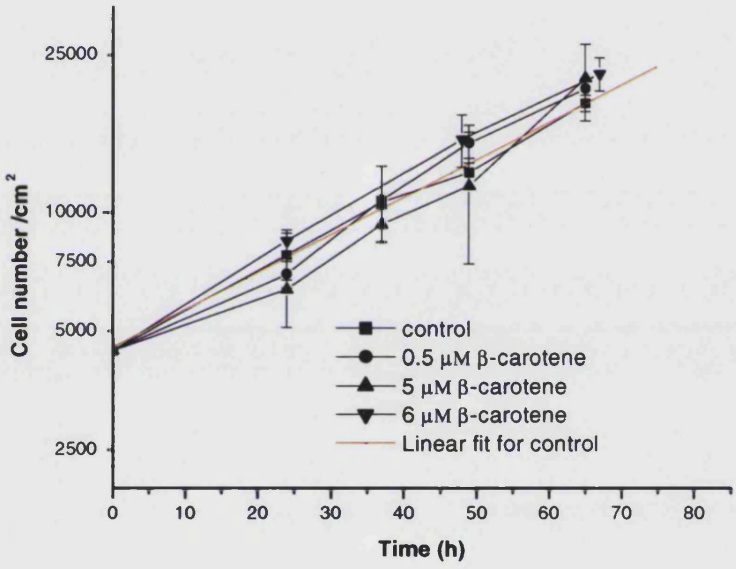


Fig. 3.4-1. Growth curve of FEK4 cells with or without β-carotene 0.5, 5 and 6 μM in the medium. Results are means +/-SD, n=3.

Lycopene is well known to induce a growth delay in cancerous cell lines (Karas *et al.*, 2000; Nara *et al.*, 2001; Ben Dor *et al.*, 2001, Prakash *et al.*, 2001) and this has been also observed in normal human fibroblasts (Levy *et al.*, 1995). We performed experiments in order to assess whether such an effect occurred in FEK4 cultures when incubated with lycopene at concentrations 0.5 and 5 μM in the medium and whether a difference between growth of cultures incubated with β-carotene or lycopene would become apparent. When the cells were incubated with 5 μM lycopene in the medium we

observed an approximately 10 hr growth delay. The cell populations incubated with 5 μM lycopene were reduced after ~36 hrs ($p=0.05$) and after ~50 hrs ($p<0.05$), when compared to populations of control cells **(Fig. 3.4-2)** or cells treated with β-carotene **(Fig. 3.4-3)**.

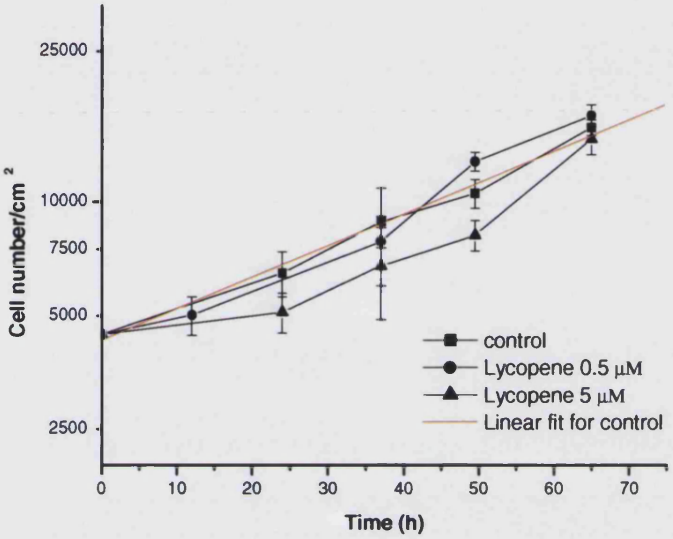


Fig. 3.4-2. Growth curve of FEK4 cells with or without lycopene 0.5 and 5 μM in medium. Results are means +/-SD, n=3.

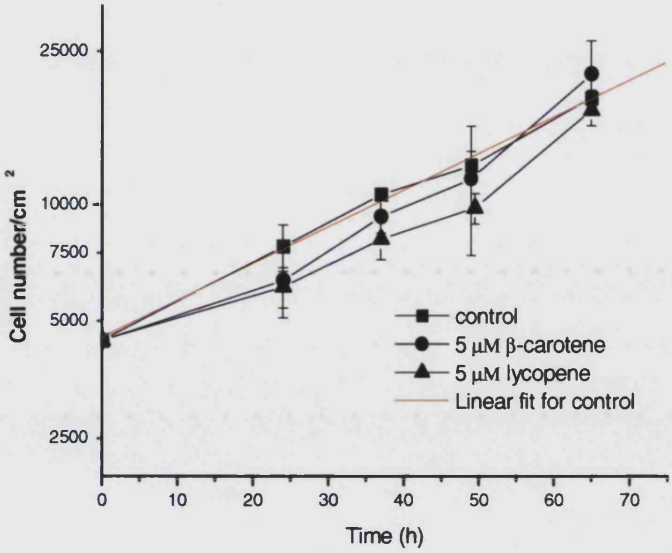


Fig. 3.4-3. Growth curve of FEK4 cells with or without β-carotene or lycopene 5 μM in the culture medium. Results are means +/-SD, n=3.

3.5 Real Time PCR LightCycler

3.5.1 Description of the LightCycler Real Time PCR

Basic Description of the Instrument. The LightCycler **(Fig. 3.5.1-1)** consists of an upper unit and a lower unit. The upper unit contains the heating coil. The lower unit contains the thermal chamber, fluorimeter, drive units, electronic boards and power supply. Hot or ambient temperature air, introduced into the thermal chamber, regulates the temperature of the sample capillaries. A heating coil heats the air, which is then fed into the chamber by a fan.

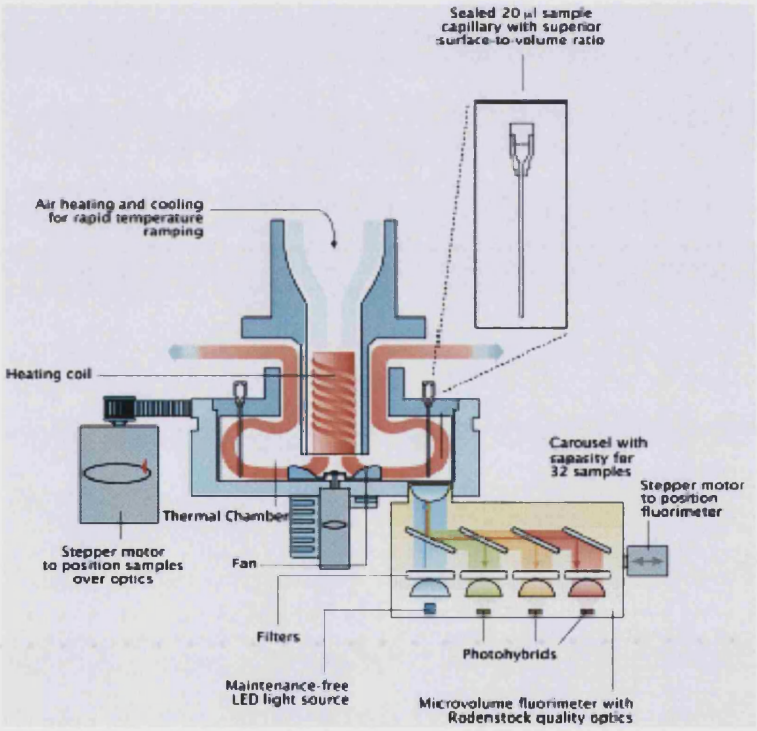


Fig. 3.5.1-1 Schematic of the LightCycler. The figure is reproduced from the *LightCycler Operator's Manual Version 3.5*

The fan ensures efficient air circulation and temperature homogeneity during the heating cycle and operates at a higher speed to ensure adequate cooling during the cooling cycle. Within the thermal chamber is the sample carousel. The carousel holds 32 samples in glass capillaries that serve as cuvettes for fluorimetric determination of the PCR products formed. The glass capillaries permit reaction volume to be 10–20 µl. Their optimised surface-to-volume ratio guarantees rapid thermal transfer within the reaction mixture. As a result, the temperature transition times during PCR are fast and the total time that is required for each cycle is approximately 20 seconds. The thermal chamber is directly connected to the optical system of the fluorimeter that is used for detection purposes. A blue diode (LED) with maximum emission of 470 nm serves as the energy source for sample excitation. Fluorescence is detected at 530 nm, 640 nm, and 710 nm with the aid of photohybrids. For measurements, a stepper motor rotates the sample carousel to position the capillary tip at the focal point of the fluorimeter optics. For online display, data are transmitted to and from a PC via a serial interface.

Programming and Run. To perform a LightCycler run, the protocol for thermocycling and fluorescence acquisition has to be programmed, i.e. the temperatures for denaturation, annealing and extension have to be determined as well as the respective times. Additionally, the location for data acquisition events needs to be specified, as well as sample information and other settings, for later data analysis. The LightCycler Run Software provides the tools for programming the experimental protocol which serves as the running pattern for multiple LightCycler runs, displays appropriate screens for performing and monitoring the LightCycler Run, loads the programmed data in the LightCycler instrument, starts and controls the machine and stores the acquired data on the PC.

Principles of Fluorimetric Online Detection. There are two different methods for online detection and evaluation of fluorimetric PCR reactions in glass capillaries. The PCR products formed during the PCR reaction may be detected via either: fluorophores that bind to all double-stranded DNA molecules regardless of sequence, e.g., the double-stranded DNA binding dye SYBR Green I, or fluorophores coupled to sequence-specific oligonucleotide hybridization probes that only detect certain PCR products.

In this study, the sequence-independent detection with SYBR Green I was used, a dye that binds specifically to double-stranded DNA (dsDNA). Its inherent fluorescence is enhanced when it binds to the minor groove of double-stranded DNA. During PCR, SYBR Green I binds to DNA products as they are synthesised. Thus, the increase in SYBR Green I fluorescence, when measured at the end of each elongation cycle, indicates the amount of PCR product formed during that cycle. The LightCycler measures the fluorimetric intensity of SYBR Green I at the end of each elongation phase. The maximal emission of DNA stained with SYBR Green I occurs at 521 nm. When a LightCycler PCR run is started, the monitor switches to the 'run screen' that displays actual fluorescence values, the development of fluorescence during the run, and the actual temperature online.

Display and Analysis of Quantification Data. For analysis of quantification data, the LightCycler Software only considers fluorescence values measured in the exponentially growing phase of the PCR amplification process. This phase is referred to as the 'log-linear' phase, because the exponential curve is transformed to a linear curve upon logarithmic plotting. Both methods used for quantification with the LightCycler Software (Fit Points Method and Second Derivative Maximum Method) refer to the log-linear phase of PCR for quantification. Analysis during log phase permits many genes (over a wide input target range) to be analysed under comparable conditions, without concern of reaching reaction plateau at different cycles (Heid *et al*, 1996). Comparable fluorescence values of different samples and standards are then used to define a standard curve, where concentrations can be allocated to the Crossing Points.

Crossing Points represent cycle numbers, at which fluorescence signal reaches an arbitrary but defined threshold value within the early exponential phase of the reaction. The Crossing Point is dependent on the starting amount of target in the sample – and is inversely related to the amounts of starting material (Higuchi *et al.*, 1993). This relationship is used for quantitative analysis with the LightCycler software.

Quantification Methods. Two different methods for quantification can be used with the LightCycler Software: the Fit Points Method and the Second Derivative Maximum Method. Both methods are generating standard curves to calculate unknown samples.

For standard curve, the standard concentrations are plotted versus the crossing points (or threshold cycles). The two methods differ in the way of threshold cycle determination.

In the Fit Points Method, the quantitative analysis of PCR data is influenced by the user, in the adjustment of the noise band and of the baseline. The Fit Points method requires a setting of baselines to a comparable level, to allow comparison of crossing point values of different samples in the same LightCycler run and between different runs. To restrict the quantification to the exponentially growing PCR part, selection of Fit Points number should be restricted to the linearly plotted phase of the PCR (in the semi-logarithmic plotting) curve.

The Second Derivative Maximum Method for quantification is automatically determining the crossing points for the individual samples. This is achieved by a software algorithm, which identifies the first turning point of the fluorescence curve, which serves as the Crossing Point in this calculation method. This turning point corresponds to the first maximum of the second derivative curve, thus explaining the name of the method. Both methods for quantification of cDNA levels are efficient. $MgCl_2$ concentration needs to be optimised for PCR efficiency, as judged by the crossing point of each PCR amplification and by a good separation of melting peaks of products and by-products. This enables fluorescence measurement at a temperature at which primer-dimers will be denatured.

Display and Analysis of Melting Curve Data. A melting curve is produced following the completion of PCR, in which the fluorescence of the samples is monitored while the temperature is increased. Briefly, a melt program that is included in all LightCycler protocols using SYBR Green I as detection fluorophore is linked to the cycling program. It contains three steps: a rapid denaturation step, a short hold at optimum annealing temperature and a slow denaturation step. During the final denaturation step, fluorescence is acquired continuously or stepwise (0.2 ^{0}C) to generate a high resolution melting curve for every sample. Due to the melting behaviour of DNA, fluorescence will decrease with the increase of temperature. In case of the SYBR Green I format, this is due to the separation of double strands and consequently the release of SYBR Green I molecules, resulting in a drop in fluorescence near the denaturation temperature. The

melting curve of a PCR product depends on GC content, length and sequence, PCR products can be distinguished by their melting curves (Gonzalez and Saiz-Jimenez, 2002; Ririe *et al.*, 1997). The LightCycler software differentiates the melting curve data and the first negative derivative (-dF/dT) is displayed versus temperature, showing the melting peak(s) for each PCR product. The area under the peak is calculated by the LightCycler data analysis software. The peak area has a relationship to the relative amount of nucleic acid. For example, in SYBR Green I experiments, the size of the peaks representing each product will show a correlation to the amount of each product.

Relative Quantification Using Melting Peak Analysis. Although there is a relationship the Peak Area and the amount of nucleic acid contributing to the peak, conclusions have to be made carefully when using this tool. Experiments are required to provide more details on this interdependency. For an appropriate approach, it is important to consider that non-specific fluorescence has to be minimised for peak comparison. One of the factors that may influence experimental results when using this method is $MgCl_2$ concentration in PCR reactions. In order to optimise $MgCl_2$ concentration for amplifications on the LightCycler to be analysed by melting curve analysis the aim is to have that concentration in the reaction mixture that will give the most robust melting peak and will not favour the formation of primer dimers. The balance of distance and ratio between peaks for the product and by products (i.e. primer-dimers), the area under peak and the height of the peak all need to be evaluated.

Real Time Fluorimeter (RTF). The Real Time Fluorimeter (RTF) allows continuous monitoring of fluorescence of one sample and can be used to adjust the fluorimeter gains (enhancement factors in the fluorescence measuring channels) at comparable levels between different runs (LightCycler Software version 3).

3.5.2 Developing the methodology

Real time PCR is a technique several fold more sensitive than Northern Blot Analysis (Higuchi *et al*, 1993; Freeman, 1999) that allows measurement of the expression of multiple genes in the same sample with accuracy. However it is a relatively new

technique which has to be carefully set up for the specific experiments to be carried out and to be controlled for possible errors (Freeman *et al*, 1999).

In order to gain some much needed relevant experience with the new technique, we initially used duplex Taqman Real Time PCR in Hoffman La Roche (Switzerland), as guests in a laboratory in which Real Time PCR had been successfully used for assessing relative gene expression. With this method (see Materials and Methods) we analysed samples for HO-1 cDNA using rDNA as an internal control from experiments on which northern Blot analysis had already been performed to enable comparison of experimental results with the two methods. With this method we also analysed experiments with FEK4 cells in different growth phases treated with or without UVA 250kJ/m^2 and 1, 2 and 3 μM concentrations of β-carotene (results not shown). However, in the case of arrested cells threshold cycle (C_t) values for the internal control, were diverse and highly indicating very low and variable levels of rDNA in the reverse transcribed RNA samples. In cell extracts from cells that are growth arrested under serum starvation specific rRNA synthesis in vitro can be suppressed by a polymerase-I transcription inhibitory activity (PIN) (Kermekchiev and Muramatsu, 1993). This activity changes under different physiological states of the cell and reaches a maximal level soon after serum depletion and disappears readily when the cells are allowed to recover in serum rich medium. Through these first experiments valuable experience was gained and also the knowledge that rRNA was not a suitable internal control for measuring HO-1 cDNA levels in FEK4 cells at different growth phases.

3.5.2.1 Melting Peaks Analysis (Light Cycler)

The first method we used for analysing samples for HO-1 mRNA/ GAPDH mRNA levels with the LightCycler was the 'melting peaks analysis'. When more than one primer sets are included in the PCR reaction mixture of samples reverse transcribed from total RNA, peak areas can be used to measure relative cDNA concentrations. In a well controlled experiment the ratio between peaks can be used for assessing the

relative concentrations of up to three genes (LightCycler manual; Bernard *et al.*, 1998; Oleastro *et al.*, 2003).

The major objective was to measure the ratio of HO-1 mRNA/GAPDH mRNA in RNA samples from FEK4 cells rather than quantitating mRNA levels and therefore we followed the approach that allows the direct measurement of the ratio of the target gene mRNA versus reference gene mRNA in the same sample.

The design of P3 and P4 primers for HO-1 had already been proved to be successful in preliminary experiments with Taqman Real Time PCR (Hoffman La Roche Laboratories) and there was a long experience in the lab with the G1 and G2 primers for GAPDH. In order to optimise the Light Cycler PCR reactions with these primers we tested for the optimum primer annealing temperatures and for optimum $MgCl_2$ concentrations for robust PCR reactions under the specific conditions. The optimum annealing temperature was 52^0C for the primers P3 and P4 and 55^0C for the primers G1 and G2. Both sets of primers worked at a satisfactory level in LightCycler PCR amplifications, at a primer annealing temperature of 52^0C. After performing LightCycler Runs of serial dilutions of HO-1 and GAPDH linearised plasmids and reverse transcribed samples, the optimum plasmid concentration for measuring HO-1 and GAPDH cDNAs in RT samples from our experiments was determined to be approximately 10^4 and 10^6 copies/μl, respectively. We tested the effect of $MgCl_2$ concentration at concentrations ranging from 2 to 6 mM on the amplification of $2.68x10^4$ copies of a *XhoI/BamHI* linearised HO-1 plasmid with the primers P3 and P4 and the optimum $MgCl_2$ concentration was found to be 3 mM. Representative LightCycler results are shown on **(Fig. 3.5.2.1-1A, B, C)**. The Tm of the HO-1 amplicon was 85.7^0C. The amplification of a *HindIII/EcoRI* linearised GAPDH plasmid was optimum at a $MgCl_2$ concentration of both 3 and 4 mM. The Tm of the PCR product was 90.5^0C (results not shown).

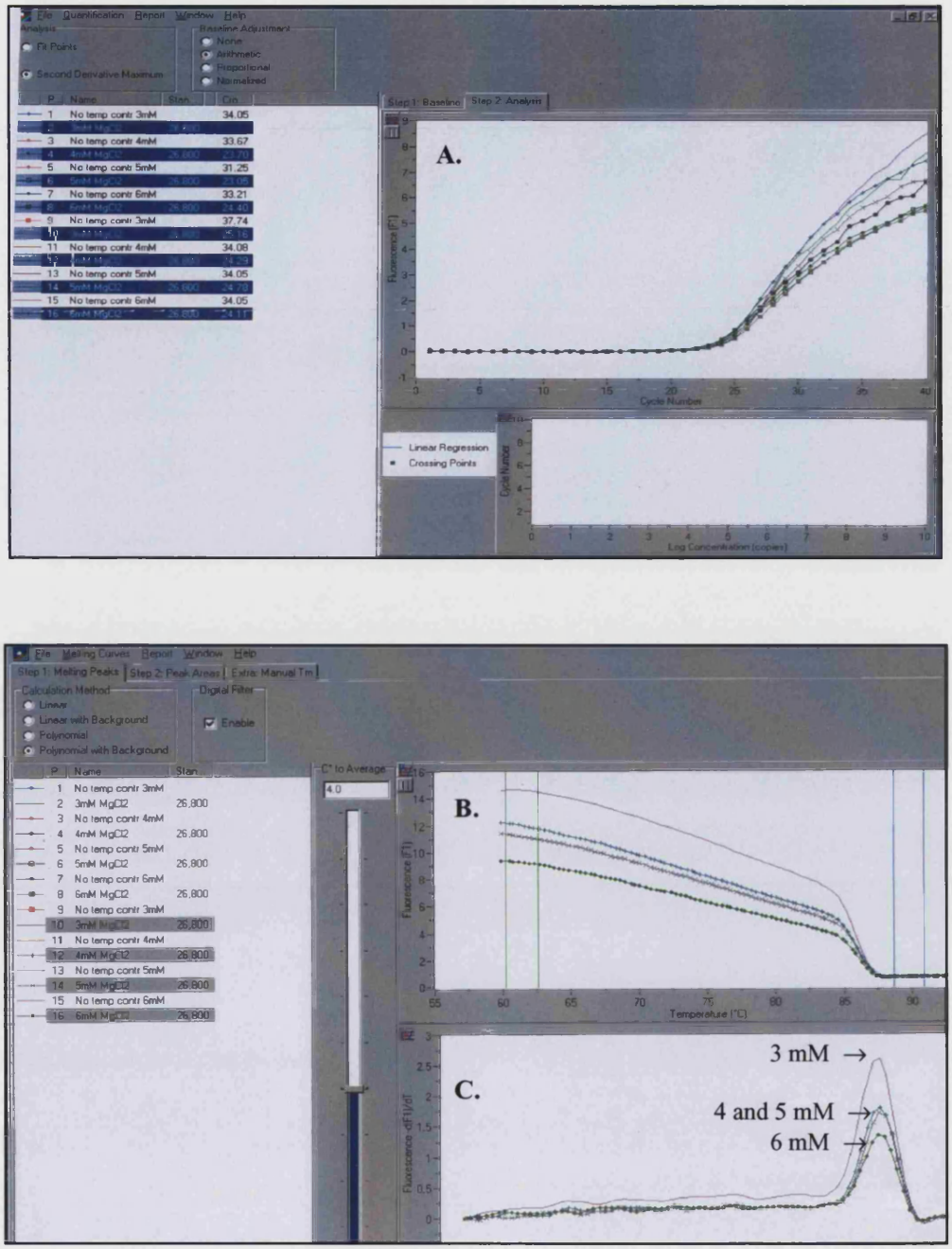


Fig. 3.5.2.1-1. Dependence of PCR amplifications on the concentration of $MgCl_2$.
(A) At $MgCl_2$ concentrations 3, 4, 5 and 6 mM the amplification of 26,800 copies of *XhoI/BamHI* linearised HO-1 plasmid was equally efficient and the crossing points were identical **(B)** Melting curves of amplifications with 3, 4, 5 and 6 mM $MgCl_2$ in the PCR reaction mixture and **(C)** their corresponding melting peaks. When 3 mM $MgCl_2$ is used, the peak is more robust than with the other $MgCl_2$ concentrations tested (4, 5 and 6 mM).

In view of these findings it should be feasible to run both reactions under the same PCR conditions (primer annealing temperature 52^0C and 3 mM $MgCl_2$). The elongation time was chosen to be adequate for the elongation of the longest amplicon (GAPDH).

Real Time PCR amplification reactions of 2 µl RT aliquots were performed with primers for HO–1 and GAPDH cDNA (primers P3, P4, G1, G2) at a final concentration of 0.5 mM each and the ratio of the two cDNAs was calculated using melting curve analysis. The PCR amplification protocol used consisted of an initial denaturation at 95^0C for 30s followed by 30 cycles of 95^0C for 0s, 52^0C for 5s, 72^0C for 8s. With this cycling protocol the PCR amplification reactions of RT samples were in the exponential phase and did not reach a reaction plateau. The experimental result showed that there is a good separation of the HO-1 and GAPDH amplicon peaks under these conditions **(Fig 3.5.2.1-2)**.

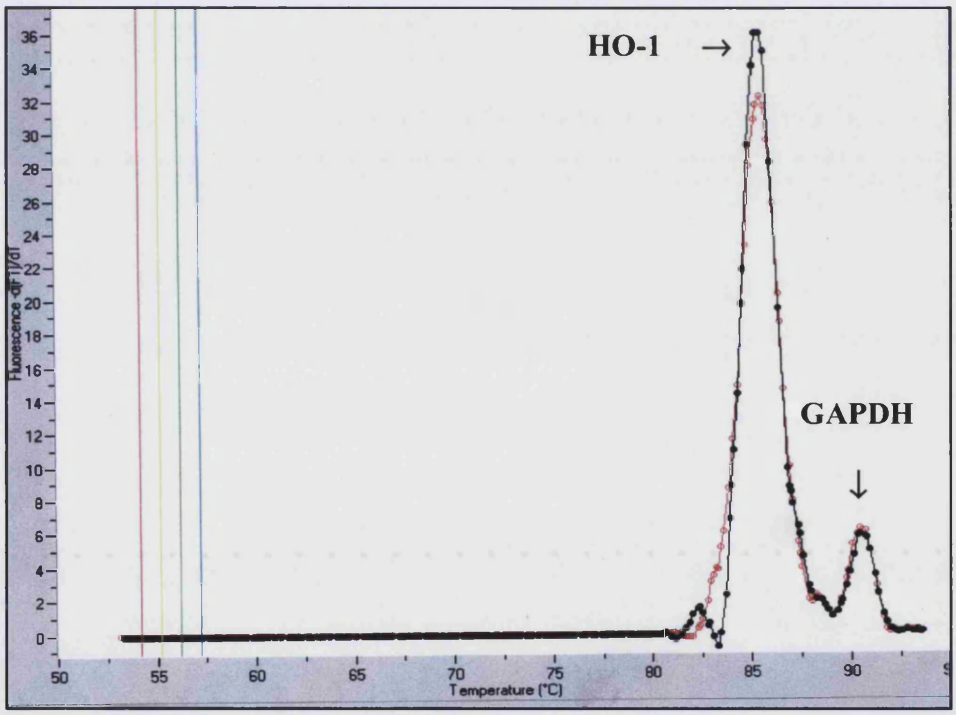


Fig. 3.5.2.1-2. HO-1 versus GAPDH ratio analysed with Melting Peaks analysis. The ratio HO-1 cDNA vs GAPDH cDNA in two RT samples from FEK4 cells treated with UVA 250kJ/m^2 and incubated for 4 h post irradiation, as seen on the LightCycler melting peaks analysis screen. The respective amplicons are distinguished by their corresponding Tm, which is 85.7^0C and 90.5^0C for the HO-1 and GAPDH amplicons respectively.

The change of the ratio of HO-1 cDNA versus GAPDH cDNA in RT samples from FEK4 cells 4 hours after UVA (250 kJ/m^2) irradiation over that in RT samples from corresponding sham irradiated controls, as seen on the melting peaks analysis screen with the LightCycler is shown on **Fig 3.5.2.1-3**. In this case, the fold increase of HO-1 cDNA (normalised over GAPDH) in the RT samples from UVA irradiated FEK4 cells was calculated to be approximately 9 fold over control values.

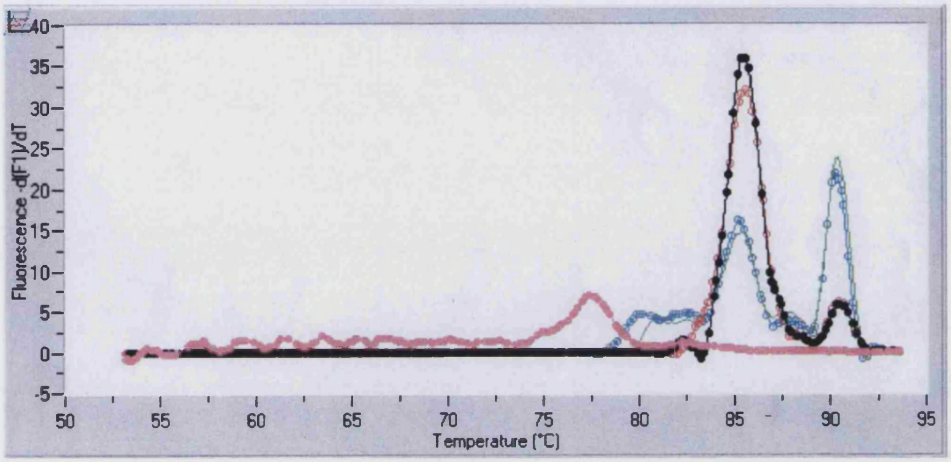


Fig. 3.5.2.1-3. The ratio of HO-1 cDNA versus GAPDH cDNA in RT samples from UVA (250 kJ/m^2) and sham irradiated FEK4 cells. The black and red lines correspond to two RT samples from UVA (250 kJ/m^2) irradiated FEK4 cells and the blue and green lines correspond to two RT samples from sham irradiated controls. The peaks corresponding to the HO-1 and GAPDH cDNA amplicons are distinguished from their melting temperatures that are 85.7^{0}C and 90.5^{0}C, respectively. In this case, the fold increase of HO-1 cDNA (normalised over GAPDH) in the RT samples from UVA irradiated FEK4 cells was calculated to be approximately 9 fold over control values.

The first LightCycler experiments were run on samples in which the fold increase of HO-1 mRNA induction by UVA had already been quantified with Northern Blot analysis (vs GAPDH) and Taqman Real time PCR (vs rRNA), to enable direct comparison of the experimental results **(Fig. 3.5.2.1-4)**. The comparison of the analysis of HO-1 mRNA accumulation 2, 4, 6, and 8 h after UVA (250 kJ/m^2) irradiation of FEK4 cells with the three methods shows a similar pattern of induction, although the fold accumulation of HO-1 mRNA vs reference gene does not compare in absolute values.

The fold induction when measured with Taqman Real time PCR is higher when compared with the same induction measured with other two methods. The difference almost certainly arises from a more accurate estimate of the very low levels of basal gene expression that will strongly affect the calculated fold increase. The melting peaks analysis with the LightCycler gave the lowest values when compared to Northern Blot analysis and Taqman. However, the results compared well with published results showing a maximum fold increase of HO-1 mRNA accumulation by UVA (250 kJ/m^2) six hours after irradiation (Applegate *et al.*, 1995b; Kvam *et al.*, 1999).

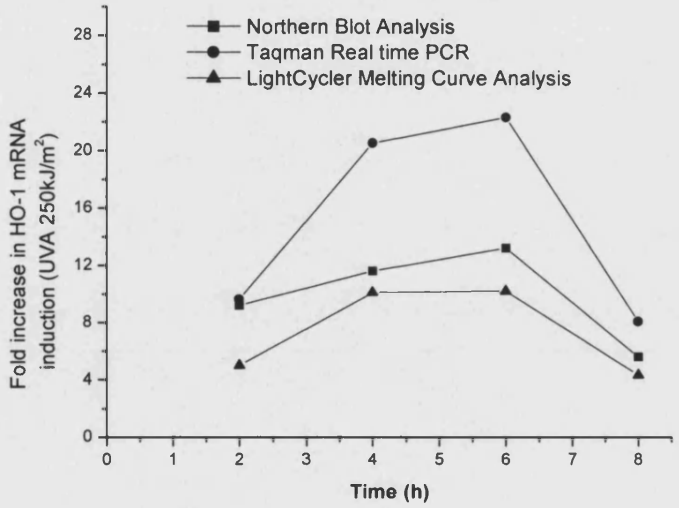


Fig. 3.5.2.1-4. HO-1 mRNA kinetics after UVA irradiation (UVA 250kJ/m^2). Fold increase of HO-1 mRNA over control values 2, 4, 6, and 8 hours after UVA irradiation (UVA 250kJ/m^2) as measured with the Taqman assay, Northern Blot Analysis and Melting peaks analysis with the LightCycler. The results shown are the mean values from two experiments, with the exception of Northern Blot Analysis (results of a single experiment).

With this method we also analysed the ratio of HO-1 cDNA vs GAPDH cDNA levels in reverse transcribed (RT) samples from experiments in which growth arrested or exponentially growing FEK4 cells had been treated with or without UVA irradiation and β-carotene. A repeated observation was that in samples where the HO-1 mRNA induction was predicted to be (or had been already shown to be with of Taqman and Northern Blot analysis) relatively high, the melting peaks analysis could not always

calculate the ratio of HO-1 cDNA /GAPDH cDNA. Such an example as seen in the melting peaks analysis screen is shown on **Fig. 3.5.2.1-5.**

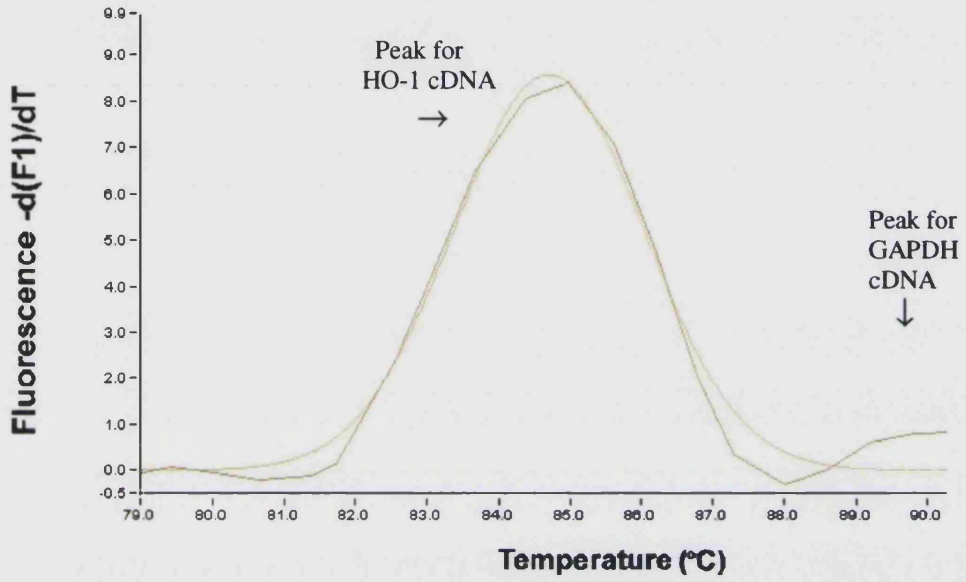


Fig. 3.5.2.1-5. The melting peaks analysis screen from a RT sample with large ratio of HO-1 cDNA/GAPDH cDNA. The melting peaks analysis screen for a RT sample from exponentially growing FEK4 cells irradiated UVA (200 kJ/m^2) following a 3 day incubation with 1 μM β-carotene in the medium. In this case, the ratio of HO-1 cDNA/GAPDH cDNA could not be quantified.

Additionally, a concern regarding the accuracy of this method for quantitation of relative gene expression is related to the fact that this is based on fluorescence measurements acquired from the final phase of PCR amplifications (Wittwer *et al.*, 1997). We also performed an experiment with HO-1: GAPDH plasmids at copy number ratios of 10:1, 5:1, 2:1 and 1:1. The range of total copy number decreased in the order 10:1, 5:1, 2:1 to 1:1 and was between $2.2x10^4$ and $11.9x10^4$ in the PCR reactions. The reactions with a higher HO-1: GAPDH ratio (10:1 and 5:1) reached a plateau whereas the reactions with a smaller HO-1: GAPDH ratio (2:1 and 10:1) did not. The results do not reflect the ratio of plasmids that were used as target templates **(Table 3.5.2.1-1)**.

For the reasons mentioned above, the decision was taken to replace the method for the quantitation of relative HO-1 mRNA levels.

Plasmid ratio	HO-1	GAPDH	HO/GAPDH	Fold increase over 1:1
HO-1: GAPDH (1:1)	41.3	6.3	6.6	1.0
HO-1: GAPDH (2:1)	43.3	4.8	9.2	1.4
HO-1: GAPDH (5:1)	51.6	4.9	10.6	1.6
HO-1: GAPDH (10:1)	50.9	3.6	14.1	2.1

Table 3.5.2.1-1. Results of Melting Peaks Analysis of PCR amplifications of HO-1 and GAPDH linearised plasmids. Melting peaks analysis of HO-1: GAPDH at ratios of 1:1, 2:1, 5:1 and 10:1. Results shown are from a single experiment.

3.5.2.2 Estimation of HO-1 and GAPDH mRNA levels with use of crossing points of PCR amplification reactions with the Second Derivative Maximum and Fit Points method (LightCycler software version 3).

The method used to assess the level of HO-1 and GAPDH cDNAs for each sample was replaced by the Second Derivative Maximum Method or the Fit Points method with use of the double-strand-specific dye SYBR Green I.

The crossing point of each sample is dependent on the starting amount of target in the sample – and is inversely related to the amounts of starting material. This method is based on the generation of a standard curve in which crossing points of PCR amplifications are allocated to serial dilutions of template DNA.

Since, intercalating dyes such a SYBR Green I bind non-specifically to DNA duplexes, non-specific amplification products such as primer-dimers can contribute to the fluorescence signal and result in inaccuracies in quantitation. We did not repeat the experiments for optimum $MgCl_2$ concentration. $MgCl_2$ was used at a concentration of 3 mM in the PCR reactions and resulted to a good separation of peaks for PCR products and non-specific PCR by-products. However, in the case of HO-1 plasmid amplification, primer-dimers were contributing to the measured fluorescence, as seen from the melting curve analysis. The melting temperatures of the non specific PCR by-products were in the range between 60^0C and 80^0C (results not shown). Therefore, fluorescence measurements in LightCycler runs for the determination of HO-1 cDNA levels in each sample were programmed to be taken at a temperature of 81^0C after the end of the elongation step (72^0C) in each PCR cycle.

In order to determine the levels of HO-1 cDNAs in RT samples we included in each LightCycler run serial dilutions of a *XhoI/BamHI* linearised HO-1 plasmid from the human heme oxygenase clone 2/10 (Keyse and Tyrrell, 1989) at copy numbers ranging from $2.7x10^3$ to $2.7x10^7$. Serial dilutions of a *HindIII/EcoR1* linearised GAPDH plasmid of the rat GAPDH gene at copy numbers ranging from $5.4x10^3$ to $5.4x10^6$ or from $5.4x10^4$ to $5.4x10^7$ were included in each LightCycler run for GAPDH cDNA quantitation in RT samples. The quantitation was based on the crossing points of the plasmid dilutions that were included in duplicate in each LightCycler run. However, in this way a large inter-run variability was observed and measurements of the same sample in different runs, using the same method, gave a different value for HO-1 and GAPDH cDNA. This happened despite the fact that the standard curve produced appeared to have an excellent regression (R^2~1). An illustrating example of the results from one of the first experiments for quantitation GAPDH levels with this method is shown on **(Fig 3.5.2.2-1)**.

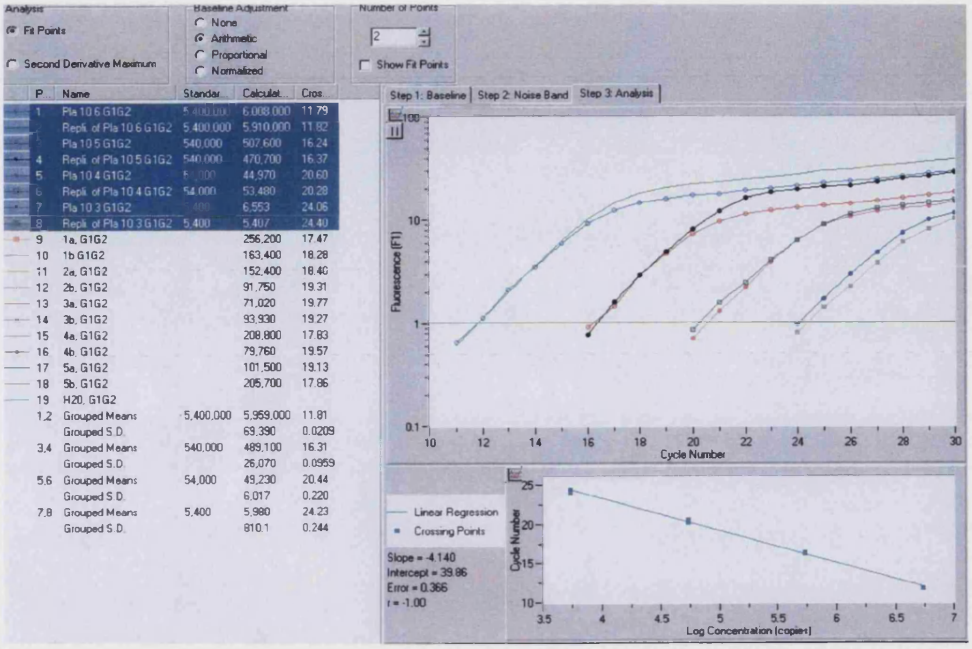


Fig. 3.5.2.2-1. Standard curve for the amplification of a rat GAPDH plasmid with primers G1 and G2. Serial dilutions of the rat GAPDH plasmid at copy numbers ranging from at copy numbers ranging from $5.4x10^3$ to $5.4x10^6$ are included in duplicate for the determination of the GAPDH c DNA content in RT samples. The analysis is carried out with the Fit Points Method [Thermal profile of PCR amplification of a 178 bp fragment of the GAPDH cDNA: a denaturation step at 95^0C was for 30 sec followed by 30 cycles of 95 for 0sec, 52 for 5sec, 72 for 8sec].

An inter-run variability for measurements with the LightCycler has been previously described as well as a variation of measurement depending on the position of the sample on the LightCycler carousel (Wilhelm *et al.*, 2000). This variation was shown to be independent of primer annealing temperatures and times as well as denaturation time.

We therefore looked for another solution and followed the approach described by Paton et al. (2000) and programmed the fluorescence gains (acquisitions) of the LightCycler to be the same at each run by using the Real Time Fluorimeter (RTF) for a control sample. The crossing points were determined with use of the 'Fit Points method'. To derive the standard curve, the cycle at which each dilution reached an arbitrary fluorescence (value

3.0) was plotted against the logarithm of the initial copy number of the plasmid. The quantitation was based on the crossing cycle of each sample, which was then extrapolated to the standard curve **(Figs. 3.5.2.2-2 and -3)**. For each sample the ratio of copy numbers was calculated. The HO-1 cDNA and GAPDH quantitation for each sample was analysed in triplicate and the profiles of each triplicate coincided reproducibly for the HO-1 cDNA, but not for the GAPDH cDNA. Consequently, the samples that were analysed with this method for estimation of HO-1 mRNA levels and normalised versus the housekeeping gene GAPDH had high errors.

To correct the problem with GAPDH amplification we changed the annealing temperature for the primers G1 and G2 to 55°C that had proved to be the optimum annealing temperature for these primers. So the standard protocol for PCR amplification of a 178bp fragment of the GAPDH cDNA G1 and G2 was changed and the new protocol consisted of a denaturation step at 95°C for 30 sec followed by 40 cycles of 95°C (0 sec), 55°C (5 sec), 72°C (7 sec).

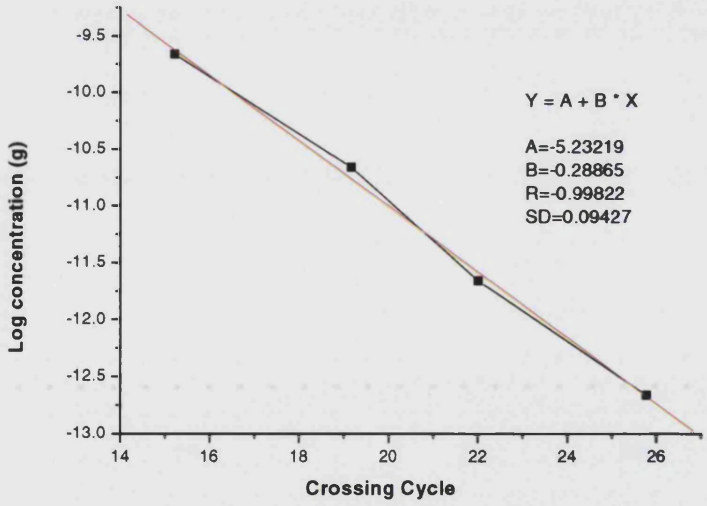


Fig. 3.5.2.2-2 Standard curve for amplification of a 74bp HO-1 amplicon (Fluorescence measurement at 81°C,Tan 52°C, 3mM $MgCl_2$) with the LightCycler. Results show means, n=3.

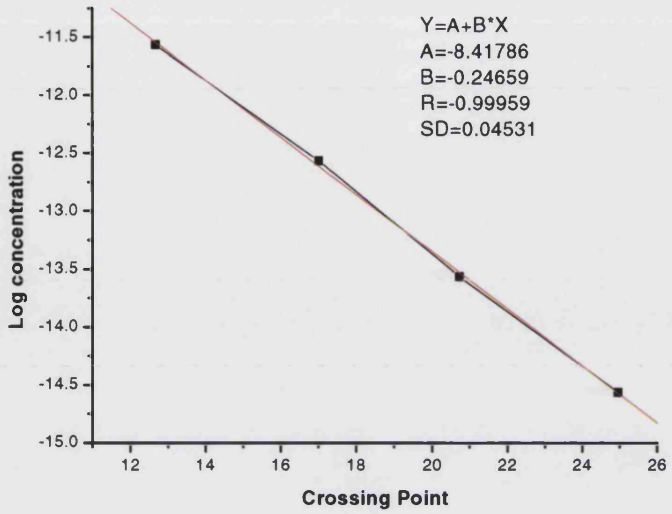


Fig. 3.5.2.2-3 Standard curve for amplification of a 178bp GAPDH amplicon (Tan 55°C, 3mM $MgCl_2$) with the LightCycler. Results show means, n=3.

However, this step did not greatly reduce the error in GAPDH quantitation with the LightCycler. We then hypothesized that the problems encountered could be due to the use of rat plasmid for deriving the standard curve for the quantification of GAPDH in reverse transcribed samples from human FEK4 cells. DNA concentration has been shown to differently affect the efficiency of PCR amplifications depending upon the source from which the DNA was extracted (Harris and Jones, 1997). The PCR amplification product when the rat plasmid is used as DNA template and the amplicon of GAPDH cDNA present in the RT samples are different in the percent GC content.

The primers G1 and G2 hybridise to the nucleotides (nts) 829-848 and 978 to 1006 of the published human GAPDH cDNA sequence (Arcari *et al.*, 1984) and to the nts 763-783 and 921-940 of the published rat GAPDH cDNA sequence (Tso *et al.*, 1985). The amplicon after PCR amplification of the rat plasmid and the human is of identical size (178bp), but the percent GC content is 52% for the rat amplicon and 57% for the human as seen from the direct comparison of the two cDNAs (Tso *et al.*, 1985). A difference in % GC content between the two amplicons is depicted in the melting peaks of the amplicon of this plasmid and the amplicon in RT samples **(Fig. 3.5.2.2-4)**.

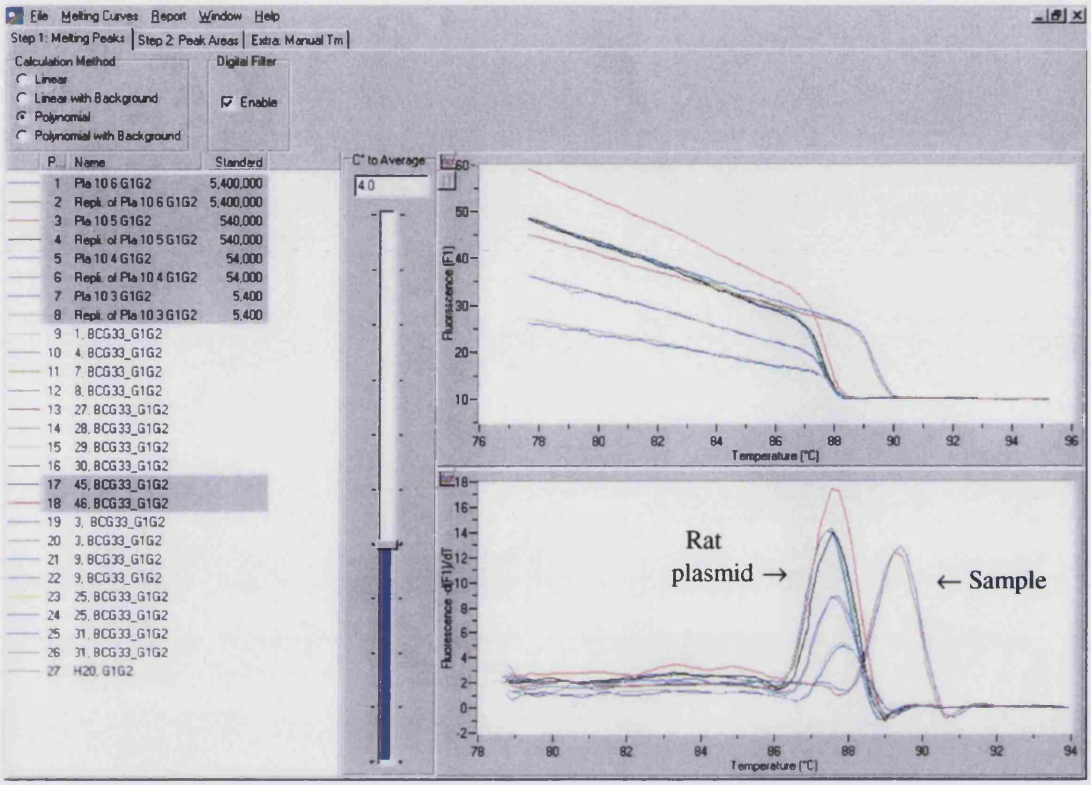


Fig. 3.5.2.2-4. Melting peaks of PCR products of the rat GAPDH plasmid and of RT samples with the primers G1 and G2. The Tm of the amplicon after PCR reactions with the primers G1 and G2 using the rat plasmid as DNA template is 87.5^{0}C. The Tm of the amplicon after PCR amplification of RT samples from human cells with the primers G1 and G2 is 89.3^{0}C.

The decision was taken to use a commercially available human GAPDH cDNA control probe to replace the rat plasmid we used to derive the standard curve and this coincided with the update of the LightCycler software (version 3.5). The new method used for GAPDH quantitation is described in the next section (section 3.5.2.3).

3.5.2.3 Measurement of crossing point of PCR amplification reactions with the LightCycler version 3.5

The LightCycler Software 3.5 includes automated Fluorescence gain adjustment and bypasses user-defined settings for fluorescence gains. Adjusting the fluorescence settings was an important step in the previous protocol to control for inter- run variability.

For this reason, the standard curves include a 'calibrator sample'. At this point the concentration of the respective cDNA is assessed in the calibrator sample. The 'calibrator is included in all subsequent runs as a standard of defined concentration. In subsequent LightCycler runs the calibrator is used to 'import' the respective standard curve that is stored on the PC. The LightCycler software then calculates the concentration of the gene in question in each sample.

The software update coincided with the change of the GAPDH cDNA we used for deriving the standard curve. We used a commercially available human GAPDH cDNA control probe (Clonetech Laboratories, USA) as template DNA at copy numbers ranging from $8.3x\ 10^4$ to 10^7 to derive the standard curve for GAPDH quantitation and used the LightCycler software version 3.5 for measurements.

These changes did minimise the error in GAPDH quantitation with use of the LightCycler and was used for following experiments. There were no further changes regarding PCR amplification protocol for measurements of HO-1 cDNA levels.

For following experiments we proceeded with the conditions and PCR reaction protocols as described in Materials and methods in section 2.4.2.2.

3.6 Modulation of UVA induced HO-1 mRNA accumulation by carotenoids

For the first experiments presented in section 3.6, the mRNA levels of HO-1 and the housekeeping gene GAPDH that was used as a control, were measured by use of Northern Blot analysis. For following experiments, real time LightCycler RT-PCR was employed for the estimations of HO-1 and GAPDH mRNA levels with the exception of one in which Taqman real time PCR was used. In the case of measurements with Taqman Real Time RT-PCR the internal control used was rRNA instead of GAPDH. The methodology employed each time is clearly stated in text and figure legends.

3.6.1 Modulation of UVA induced HO-1 mRNA accumulation by β-carotene.

UVA (100 and 250 kJ/m^2) induces HO-1 mRNA accumulation in FEK4 cells at levels that permit monitoring its modulation by carotenoids. As shown with use of Northern Blot Analysis, β-carotene modulates this induction at medium concentrations of 0.07, 0.8, 2.3, 8.0 and 21.0 μM in a different manner when the FEK4 monolayers are irradiated at a UVA dose of 100 or 250 kJ/m^2. At a UVA dose of 100 kJ/m^2 and at all tested concentrations except for the highest (21 μM) β-carotene appears to be only slightly modulating the HO-1 mRNA levels ($p>0.05$), if at all **(Fig. 3.6.1-1)**. However, at a concentration of 21 μM β-carotene, significantly suppressed the UVA induced HO-1 mRNA accumulation ($p<0.05$). On the other hand, at a UVA dose of 250 kJ/m^2 β-carotene shows a modulation that is clearly β-carotene concentration dependent **(Fig. 3.6.1-2)**. At a β-carotene concentration of 0.2 μM, there was a significant enhancement of UVA induced HO-1 mRNA accumulation when compared with the irradiated control in the absence of β-carotene ($p<0.05$). However, at the highest concentrations tested (2.3, 8.0 and 21.0 μM) β-carotene exhibits a significant dose-dependent decrease of UVA-induced HO-1 mRNA levels. At the two highest concentrations (8.0 and 21.0 μM) tested, the HO-1 mRNA levels are lowered to the levels of sham-irradiated FEK4. It

should also be noted that the difference between the level of suppression of UVA induced HO-1 activation at 2.3 μM β-carotene versus 8 and 21 μM is significant ($p<0.05$). It appears that this effect is specific for HO-1 and that the down regulation of HO-1 is not due to a general suppression of gene expression in the cell by β-carotene as GAPDH levels remain unaffected by the test agents.

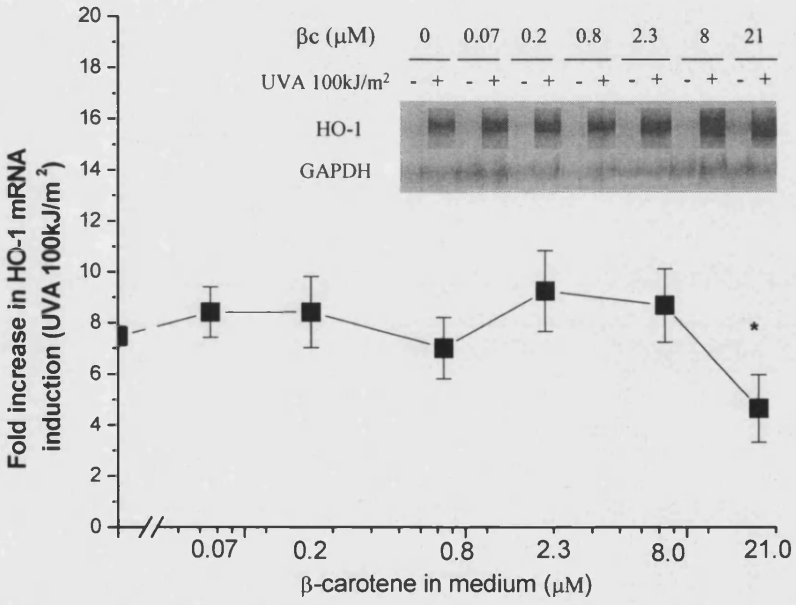


Fig. 3.6.1-1. Modulation of UVA induced HO-1 mRNA accumulation by β-carotene. The modulation of UVA induced HO-1 mRNA levels (UVA 100 kJ/m^2) normalised over GAPDH mRNA in FEK4 by 0.07, 0.2, 0.8, 2.3, 8.0 and 21 μM β-carotene. Results are means +/-SD, n=3-4. The value for irradiated control in the absence of β-carotene is 7.47±0.39. A typical Northern Blot of a single experiment is included. *: Significance level when compared with the irradiated control in the absence of β-carotene, $p<0.05$.

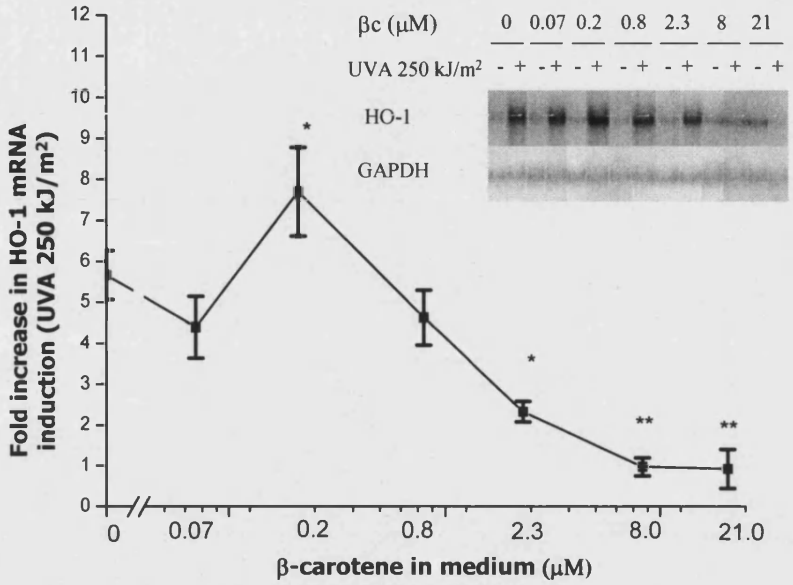


Fig. 3.6.1-2. Modulation of UVA induced HO-1mRNA accumulation by β-carotene. The modulation of UVA induced HO-1 mRNA levels (UVA 250 kJ/m^2) normalised over GAPDH mRNA in FEK4 by 0.07, 0.2, 0.8, 2.3, 8.0 and 21 μM β-carotene. Results are means +/-SD, n=3-4. The value for irradiated control in the absence of β-carotene is 5.7±0.6. A typical Northern Blot of a single experiment is included. *: Significance level when compared with the irradiated control in the absence of β-carotene, $p<0.05$. **: Significance level when compared with the irradiated control in the absence of β-carotene $p<0.005$.

We also performed Northern Blot analysis of FEK4 cells treated identically without including the extensive washing step (see Materials and Methods), to test for the importance of this washing step when measuring HO-1 mRNA accumulation. The values for UVA induced HO-1 mRNA levels over control obtained from two experiments for measuring the modulation UVA (250 kJ/m^2) induced HO-1 mRNA by 0.07, 0.2, 0.8, 2.3, 8.0 and 21 μM β-carotene performed in this way **(Fig. 3.6.1-3)** were different from those with extensive washing **(Fig. 3.6.1-2).** It appears that β-carotene interferes at a variable extent with observations on the modulation of UVA induced HO-1 gene expression, when not prevented from sticking to the cell surface by the extensive washing step, which discouraged us to continue with this protocol.

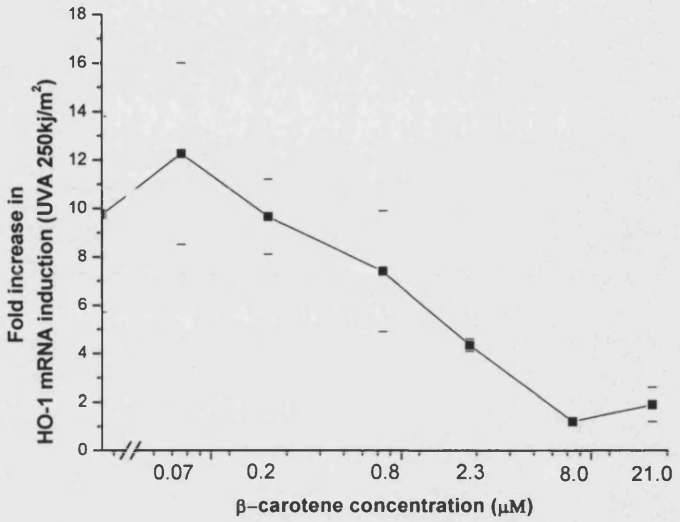


Fig. 3.6.1-3. Modulation of UVA induced HO-1 mRNA accumulation by β-carotene in FEK4 monolayers washed once with PBS. The modulation of UVA induced HO-1 mRNA levels (UVA 250 kJ/m^2) normalised over GAPDH mRNA in FEK4 by 0.07, 0.2, 0.8, 2.3, 8.0 and 21 μM β-carotene, as measured with Northern Blot Analysis when the extensive washing step was excluded. Results shown are the means of two experiments; the minimum and maximum values are represented as -.

The modulation of UVA induced HO-1 mRNA levels (UVA 250 kJ/m^2) in FEK4 by 0.07, 0.2, 0.8, 2.3, 8.0 and 21 μM β-carotene was also measured with a 5' nuclease PCR assay using the ABI PRISM 7700 Sequence detector (Taqman) after reverse transcription of each total RNA sample. The internal control used was rRNA. The results obtained are similar although fewer samples were included in the measurements **(Fig.3.6.1-4)**.

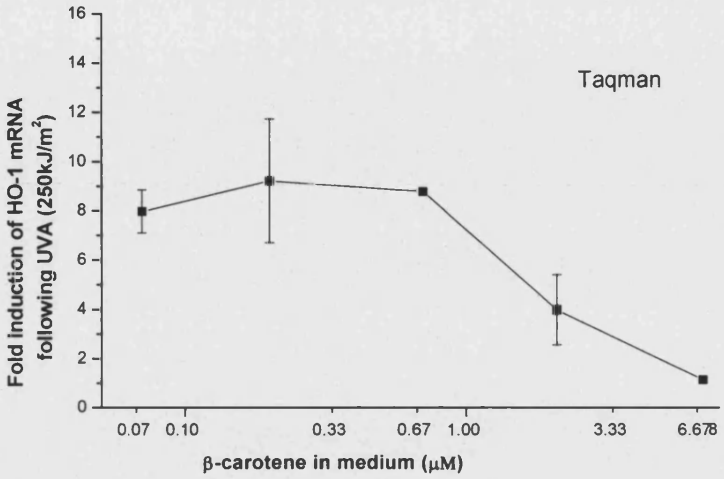


Fig. 3.6.1-4. The modulation of HO-1 mRNA induction in FEK4 UVA irradiated at 250 kJ/m^2 by 0.07, 0.2, 0.8, 2.3 and 8.0 μM β-carotene, as measured with Taqman. Results show means +/- SD, *n*=1-3. (Each of the values is the mean of triplicate measurements).

In experiments carried out with Real Time RT-PCR (LightCycler) (Fit Points method) to measure the modulation of UVA (100, 200, 250 kJ/m^2) induced HO-1 gene expression by 1 μM β-carotene no suppression of UVA induced HO-1 mRNA was observed at any of the UVA doses tested (100, 200, 250 kJ/m^2) **(Fig. 3.6.1-5)**. This is in accordance with our previous observations **(Figs. 3.6.1.-1, -2)**. It should be noted, however, that in this experiment the HO-1 measurements were normalised against GAPDH levels, which were calculated by use of a standard curve derived from serial dilutions of rat GAPDH plasmid that introduced errors in normalised measurements.

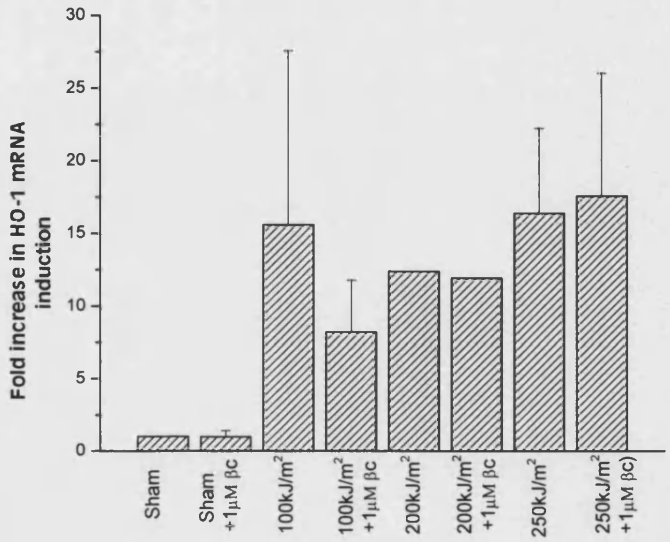


Fig. 3.6.1-5. Modulation of UVA induced HO-1 mRNA accumulation by β-carotene. The modulation of UVA induced HO-1 mRNA levels (UVA 100, 200 and 250 kJ/m^2) normalised over GAPDH mRNA in FEK4 by 1 μM β-carotene. Results are means +SD, n=3-4, When no error bars are shown the results are means of two experiments.

In additional experiments that were carried out with Real Time RT-PCR (LightCycler) [Analysis of crossing point of PCR amplifications] to measure HO-1 mRNA levels it was demonstrated that at a concentration of 5 μM β-carotene, the suppression of HO-1 mRNA accumulation increases with increasing UVA dose applied. A very significant suppression of UVA induced HO-1 expression ($p<0.005$) occurs at the highest UVA doses tested (200 and 250 kJ/m^2) **(Fig. 3.6.1-6)**.

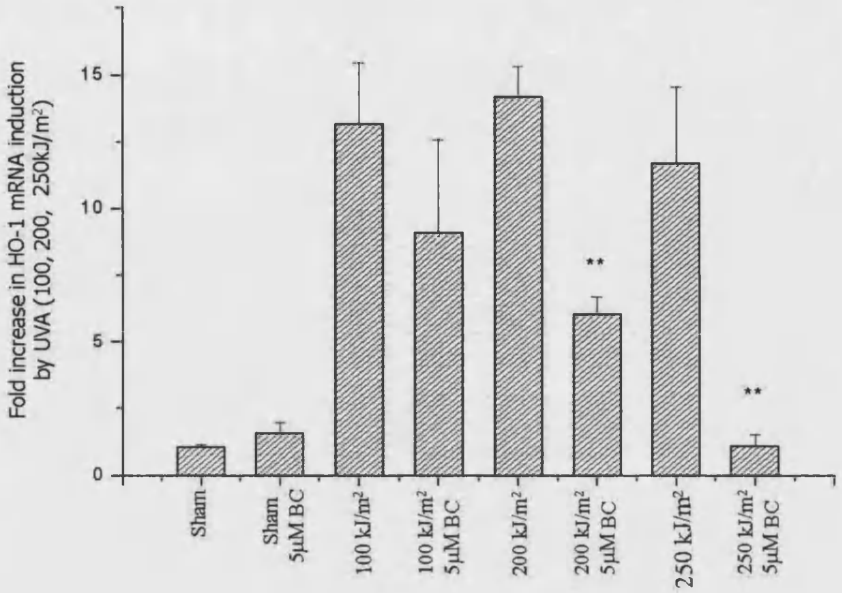


Fig. 3.6.1-6. HO-1 mRNA accumulation in FEK4 cells after UVA (100, 200, 250 kJ/m^2) +/- 5 μM β-carotene. The modulation of UVA induced HO-1 mRNA levels (normalised over GAPDH mRNA) by 5 μM β-carotene in the medium at different UVA doses (100, 200, 250 kJ/m^2), as measured with Real Time PCR (LightCycler) Results are means +SD, n=3-4.**: Significance level when compared with the irradiated control in the absence of β-carotene, $p<0.005$.

3.6.2 Modulation of UVA induced HO-1 mRNA accumulation by lycopene.

We have measured the modulation of HO-1 mRNA accumulation induced by UVA (50, 100 and 250 kJ/m^2) in exponentially growing FEK4 cells by 0.1, 0.3, 1.2, 3.0 and 8.8 μM lycopene using the LightCycler Real-Time PCR [Analysis of crossing point of PCR amplifications]. All results were normalised against the housekeeping gene GAPDH. At a UVA dose of 50 kJ/m^2 lycopene suppressed significantly the UVA induced HO-1 mRNA accumulation only at the lowest lycopene dose tested (0.1 μM) **(Fig. 3.6.2-1).** However, the observed lack of suppression of UVA (50 kJ/m^2) induced HO-1 mRNA levels by 0.3, 1.2, 3.0 and 8.8 μM lycopene is most probably due to the low levels of induction of HO-1 mRNA accumulation by a UVA dose of 50 kJ/m^2 over sham irradiated controls. The fold induction of HO-1 mRNA accumulation by this UVA dose (50 kJ/m^2) was approximately 4 fold for the vehicle treated controls.

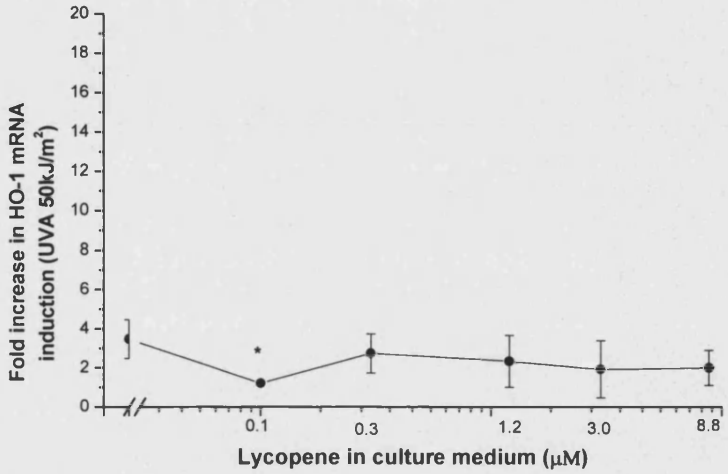


Fig. 3.6.2-1. Modulation of UVA (50 kJ/m^2) induced HO-1 mRNA accumulation by lycopene. The modulation of UVA induced HO-1 mRNA levels (UVA 50 kJ/m^2) normalised over GAPDH mRNA in FEK4 by 0.1, 0.3, 1.2, 3.0 and 8.8 μM lycopene, as measured with RealTime PCR (LightCycler). Results are means +/- SD, *n*=3-4. *: Significance level when compared with the irradiated control in the absence of lycopene, $p<0.05$.

At a UVA fluence of 100 kJ/m^2 lycopene significantly suppressed the UVA induced HO-1 mRNA accumulation at all medium concentrations of lycopene tested in exponentially growing FEK4 cells **(Fig 3.6.2-2)**. This effect is markedly different to the observed effect of β-carotene on the modulation of UVA (100 kJ/m^2) induced HO-1 mRNA levels that is presented in **(Fig 3.6.1-1)**.

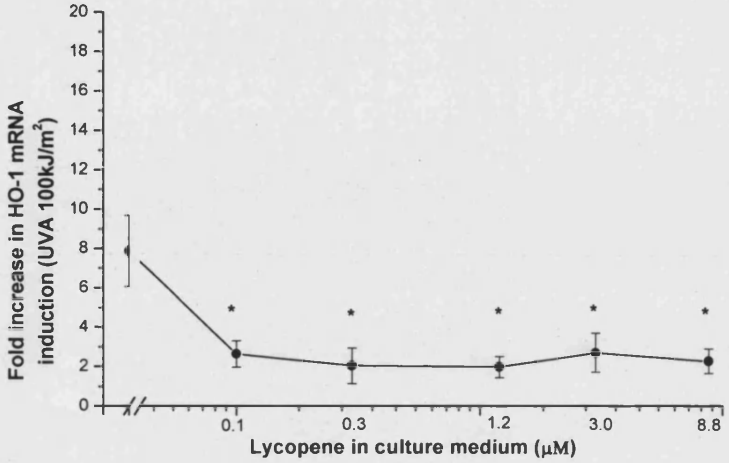


Fig. 3.6.2-2. Modulation of UVA (100 kJ/m^2) induced HO-1 mRNA accumulation by lycopene. The modulation of UVA induced HO-1 mRNA levels (UVA 100 kJ/m^2) normalised over GAPDH mRNA in FEK4 by 0.1, 0.3, 1.2, 3.0 and 8.8 μM lycopene, as measured with RealTime PCR (LightCycler). Results are means +/- SD, *n*=3-4. *: Significance level when compared with the irradiated control in the absence of lycopene, $p<0.05$.

At a UVA fluence of 250 kJ/m^2 lycopene significantly suppressed the UVA induced HO-1 mRNA accumulation when present at 0.1, 0.3, and 1.2 μM concentrations in the medium **(Fig. 3.6.2-3).** The effect was not significant at the highest lycopene dose tested (3.0 μM) ($p>0.05$), exhibiting a different pattern of suppression of UVA induced HO-1 gene expression when compared to the suppression afforded by β-carotene, which is clearly concentration dependent at this UVA dose (250 kJ/m^2) **(Fig. 3.6.1-2)**.

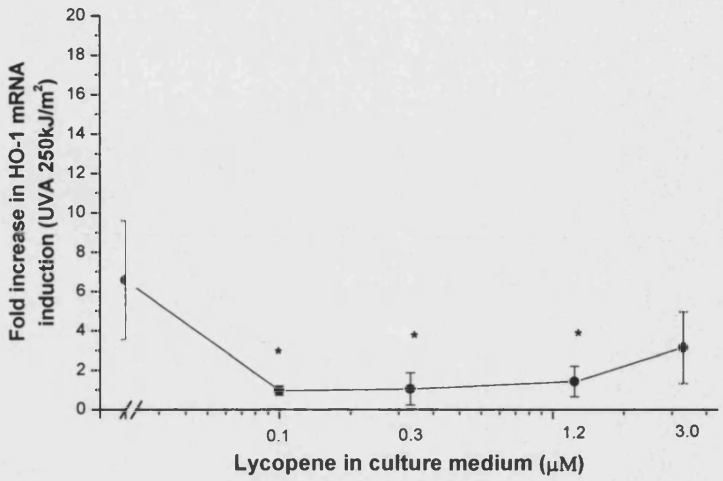


Fig. 3.6.2-3. Modulation of UVA (250 kJ/m^2) induced HO-1 mRNA accumulation by lycopene. The modulation of UVA induced HO-1 mRNA levels (UVA 250 kJ/m^2) normalised over GAPDH mRNA in FEK4 by 0.1, 0.3, 1.2 and 3.0 μM lycopene, as measured with RealTime PCR (LightCycler). Results are means +/- SD, *n*=3-4. *: Significance level when compared with the irradiated control in the absence of lycopene, $p<0.05$.

It is noteworthy, though, that lycopene at medium concentrations of 0.1, 0.3, 3.0 and 8.8 μM significantly ($p<0.05$) induced HO-1 mRNA accumulation when compared to HO-1 mRNA levels in vehicle treated cells (**Fig. 3.6.2-4**). The maximum level of induction of HO-1 mRNA in FEK4 cells by lycopene was observed after incubation of cells with the highest lycopene concentration tested (8.8 μM) and was approximately 6 fold over control values.

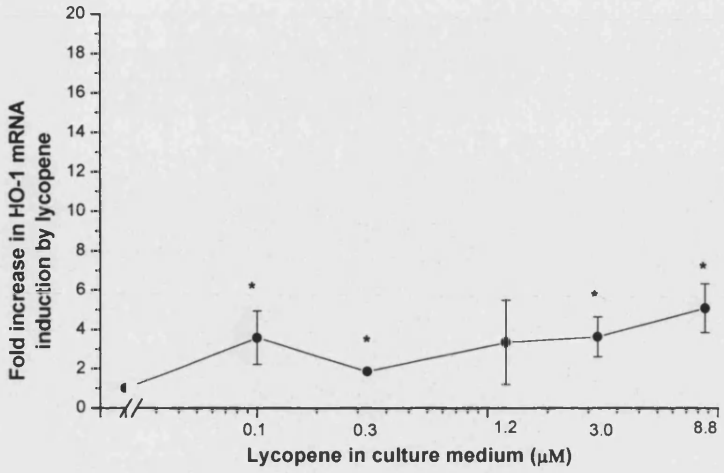


Fig. 3.6.2-4. Modulation of HO-1 mRNA accumulation by lycopene. The modulation of HO-1 mRNA levels normalised over GAPDH mRNA in FEK4 incubated with 0.1, 0.3, 1.2, 3.0 and 8.8 μM lycopene, as measured with RealTime PCR (LightCycler). Results are means +/- SD, *n*=3-4. *: Significance level when compared with controls in the absence of lycopene, $p<0.05$.

4 DISCUSSION

Previous studies have demonstrated not only that singlet oxygen is involved in UVA activation of genes, but that pure singlet oxygen generators strongly activate the expression of a number of genes (for review see Ryter and Tyrrell, 1998). Evidence exists for singlet oxygen involvement in the UVA mediated activation of HO-1 gene expression (Basu-Modak and Tyrrell, 1993). Carotenoids are powerful singlet oxygen quenchers and have been shown to quench singlet oxygen at rates approaching diffusion control in organic solvents (Edge and Truscott, 1999). Both β-carotene and lycopene suppress the UVA induced HO-1 mRNA accumulation **(Figs. 3.6.1-2, 3.6.2-2, -3)** and the possibility that they exert this effect through their activity as singlet oxygen quenchers is feasible. However, given that β-carotene, a provitamin A carotenoid, suppresses the UVA induced HO-1 mRNA accumulation as a function of concentration in the medium and UVA dose applied to the cell cultures, an alternative hypothesis that includes retinoid signaling and nuclear receptor activation could be considered, especially for β-carotene.

Under the conditions used, a physiological dose of UVA (250 kJ/m^2) induces HO-1 mRNA accumulation in FEK4 cells several fold over control values. This induction is suppressed when exponentially growing FEK4 cells are incubated for three days with

0.07, 0.2, 0.8, 2.3, 8.0 or 21.0 μM β-carotene in the medium prior to irradiation in a concentration dependent manner **(Fig. 3.6.1-2)**. This suppression is very significant when the concentration of β-carotene in the medium exceeds 4.7 μM, the estimated carotenoid content in human skin (Peng *et al.*, 1995). A contrasting lack of suppression was observed when exponentially growing FEK4 cells were incubated with the above concentrations of β-carotene in the medium under identical conditions and then UVA irradiated with a lower dose of 100 kJ/m^2. In this case, the UVA (100 kJ/m^2) induced HO-1 mRNA levels were not suppressed, with the exception of the highest β-carotene concentration in the medium (21 μM) **(Fig. 3.6.1-1)**.

The dependence of the modulation of UVA induced HO-1 gene expression by β-carotene on the UVA dose applied to the cell cultures was also observed when FEK4 cells were incubated with 5 μM β-carotene in the medium for three days and irradiated with three different UVA doses. At a UVA dose of 100 kJ/m^2 no suppression of the UVA induced HO-1 mRNA gene expression was observed, whereas the suppression of UVA induced HO-1 mRNA was very significant ($p<0.005$) for the higher UVA doses tested (200 and 250 kJ/m^2) **(Fig 3.6.1-6)**.

On the other hand, when exponentially growing FEK4 cells were incubated for three days with lycopene at concentrations of 0.1, 0.3, 1.2, 3.0 and 8.8 μM in the medium the UVA (100 kJ/m^2) induced HO-1 mRNA levels were suppressed and there was no concentration dependence in the observed effect **(Fig. 3.6.2-2)**. The UVA (250 kJ/m^2) induced HO-1 mRNA accumulation was suppressed following a three day incubation of FEK4 cells with 0.1, 0.3, 1.2 μM lycopene in the medium but not after a three day incubation of cells with a higher concentration of lycopene in the medium (3 μM) **(Fig. 3.6.2-3)**.

Under the conditions used the pattern of modulation of UVA induced HO-1 mRNA accumulation by lycopene and β-carotene is rather different and possibly suggesting different modes of action for each carotenoid.

The data presented here **(Figs. 3.6.1 -2, -6 and 3.6.2-2, -3)** could be interpreted either as an indication of $^1O_2$ quenching by the two carotenoids or, alternatively, as an indication of activation of nuclear receptors, especially by β-carotene. Which mechanism is more likely involved in the consequent suppression of UVA mediated oxidative stress by β-carotene and lycopene is an issue open to discussion that clearly remains to be elucidated. However, in view of the experimental results obtained from this study a singlet oxygen quenching appears to be a more likely mode of action for lycopene and nuclear receptor activation is probably the best way to explain the suppression of UVA induced HO-1 mRNA levels by β-carotene.

If $^1O_2$ quenching by the carotenoids were regarded as the sole mechanism by which the carotenoids suppress the UVA induced HO-1 suppression, the lack of suppression at a UVA dose of 100 kJ/m^2 by β-carotene at concentrations in the medium from 0.07 to 8 μM and the contrasting suppression by lycopene at similar concentrations in the medium (0.1- 8.8 μM) would be difficult to explain. This striking difference is further underlined if the increased β-carotene cellular levels relative to the cellular levels of lycopene at the time of UVA irradiation **(Figs. 3.1.2.-1 and 3.3.1-1)** are taken into account.

On the other hand, substantial evidence exists for the possibility of activation of RARs and RXRs by metabolites of β-carotene (Ponnamperuma *et al.*, 2000; Liu *et al.* 2000; Wang *et al.,* 1999b) and although ongoing research is tentatively addressing this issue regarding metabolites (or possible metabolites) of other carotenoids including lycopene (Ben-Dor *et al.*, 2001; Stahl *et al.*, 2000), to date no such metabolite of lycopene has been found. Regarding the mechanism of action of β-carotene in our system, the activation of the RAR/RXR and PPAR/RXR heterodimers cannot be excluded. The activation of the PPARα/RXR heterodimer accounts for regulation of β- and ω-oxidation pathways and for negative interference with the AP-1, STAT and NF-κB signalling pathways (for review see Chinetti *et al*, 2000), as well as for regulation of vitamin A homeostasis through the transcriptional control of cellular retinol binding protein (CRBP) II (Suruga *et al.,* 1999). A mechanism similar to the β-oxidation of fatty acids has been proposed for retinoic acid (RA) formation from assymetric cleavage of

β-carotene and the intermediates of such a pathway (Wang *et al.*, 1996), apocarotenals, have been detected in our system in sham and UVA irradiated FEK4 cells.

Furthermore, cyclooxygenase metabolites are natural ligands for PPARs (Kliewer *et al.*, 1999) and their level increases with increasing UVA dose (Hanson and DeLeo, 1989; 1990; Bachelor *et al.*, 2002). In the PPAR/RXR heterodimer both receptors are independently and synergistically activated by their responsive ligands (Desvergne and Wahli, 1999). Putative AP-1 binding sites are present in the promoter regions of numerous UVA activated genes and the involvement of *c-fos* and *c-jun* in the activation of such elements has been clearly demonstrated for HO-1 (Camhi *et al.*, 1998). At present it is not known whether UVA induction of HO-1 is driven by AP-1. However, an increase of *c-fos* and *c-jun* by UVA at the mRNA level has been demonstrated in FEK4 cells (Soriani *et al.*, 2000). If the observed effects of β-carotene on UVA induced HO-1 mRNA were regulated by the PPAR/RXR heterodimer, then the increase of cyclooxygenase metabolites with increasing UVA dose may lead to an increased activation of the PPAR/RXR heterodimer and an increased suppression of the HO-1 mRNA levels with increasing UVA doses, in the presence of 9-cis retinoic acid, which is a ligand for RXRs (Heyman *et al*, 1992). To date, the involvement of the PPAR/RXR heterodimer in the mediation of the effects of β-carotene in vivo has not been examined.

Formation of apocarotenals, the products of chemical or enzymatic (Wang *et al*, 1996; Kiefer *et al.*, 2001) asymmetric cleavage of β-carotene, were monitored in our system. When exponentially growing FEK4 cells were incubated with 0.3, 0.9, 2.5, 9 and 23 μM β-carotene for three days, apocarotenal formation in cells was considerable (approx. 20 pmol/million cells) at the higher concentrations **(Fig 3.1.2-2)** where the suppression of UVA (250 kJ/m^2) induced HO-1 gene activation by β-carotene peaked **(Fig 3.6.1-2)**.

β-carotene is a provitamin A carotenoid so that its biological activity could result from its conversion to retinol or retinoid metabolites (Rock et al, 1997; IARC Working Group for the Evaluation of Cancer, 1998). In the experiments presented here, no retinol was detected either in the cell culture medium or in the cell pellets. However, when 3 μM of β-carotene dissolved in THF was added to the growth medium of mouse embyonic fibroblasts (BALBc/c 3T3 cells) (Wei *et al.*, 1998). It is possible that

interspecies variation could account for this difference as rodents exhibit different patterns of β-carotene metabolism (Rock, 1997).

Also the isomerisation pattern for both lycopene and β-carotene was monitored in the experiments. In our system, cellular β-carotene isomerised to *cis*-β-carotene and the level of the cis isomer was in the range from 4-7 pmol/million cells when exponentially growing FEK4 cells were incubated for three days with all-trans β-carotene at initial concentrations of 0.3, 0.9, 2.5, 9 and 23 μM in the medium. When seen as a percentage of the total cellular β-carotene the isomerisation extent ranged from 4 to 17% **(Fig. 3.1.2-2)**. It has been demonstrated (Wang *et al.*, 1994; Herbuterne, *et al.*, 1995) that 9-cis-β-carotene can serve as a precursor of 9-cis-retinoic acid. We did not specify the exact isomerisation(s) involved, but formation of 9-cis-β-carotene is, at least to an extent, a likely event.

On the day of UVA irradiation of exponentially growing FEK4 cells the major lycopene isomer detected in cells following a three day incubation with all-trans-lycopene was the all- trans lycopene isomer that accounted for 82-100% of total lycopene in cells. The 5-cis-lycopene isomer was also detected and accounted for ~ 5% of total lycopene in cells **(Fig. 3.3.1-2).** However, to date, although 2-13 different lycopene isomers have been detected in plasma and 14-18 in human tissues, the biological relevance of cis-isomers of lycopene remains as yet unknown. The relevant knowledge is limited to indications that cis isomeric form is more bioavailable than the trans (Re *et al.*, 2001, For reviews see Clinton, 1998; Rao and Agarwal, 1999).

Overall, media β-carotene concentrations do not necessarily reflect cellular content in β-carotene, as the uptake of β-carotene by cells in culture depends not only on the delivery method used **(Figs. 3.1.1-1, -2)**, cell line and size (Wei et *al.*, 1998, Iftikhar *et al.*, 1996; Palozza, 2001*)*, but also on seeding density (Iftikhar, 1996) and growth phase **(Figs. 3.1.1-6, 3.1.2-4, 3.1.3-1).**

The reasons for the differences observed in uptake of β-carotene by different cell lines has not been established. Two distinct mechanisms have been proposed in the literature.

One involves energy independent passive transport of β-carotene across the cellular membrane and the other involves metabolically driven endocytosis (Scita *et al.*, 1992; Grolier *et al.*, 1992). It has been suggested that the increased uptake of β-carotene (Kennedy and Krinsky, 1994; Palozza *et al.*, 1996) by tumor cells might be due to increased rate of endocytosis (Schwartz *et al.*, 1991).

The kinetics of carotenoid uptake by FEK4 cells were established in this study with two delivery systems (THF or THF/EtOH) and THF was selected as the optimum carrier for β-carotene to the cultured cells. We used THF to deliver β-carotene to FEK4 cells in culture and the maximum cellular β-carotene concentration achieved after a three day incubation of FEK4 cells with the carotenoid in the medium was 300 pmol/10^6cells **(Fig. 3.1.2-4)**. This cellular concentration is equivalent to ~12 nmol/g wet tissue (IARC Working Group for the Evaluation of Cancer, 1998). With THF as the delivery system in mouse embryo fibroblasts (C3H/10T1/2) an intracellular β-carotene level of 430 pmol/10^6cells was measured after a 24h treatment with 10 μM β-carotene (Cooney *et al.*, 1993). A 24 hour incubation of WiDr cells with 50 μM β-carotene resulted in a cellular concentration of 130 pmol/million cells (Palozza *et al.*, 2001). A cellular level of 80 pmol/million cells was obtained after a 48h incubation of Chinese hamster ovary cells with 0.07 μM β-carotene (Stich and Dunn, 1986).

Incubation of FEK4 cells with lycopene in the medium resulted in intracellular levels that were several fold lower than those following an incubation with β-carotene in the medium. The maximum level observed after a 3-day incubation of FEK4 cells with 6.7 μM lycopene in the medium was ~75 pmol lycopene/million cells **(Fig. 3.3.1-1)**, whereas after under comparable conditions the cellular concentration of β-carotene was ~180 pmol/million cells **(Fig. 3.1.2-1)**. A similar finding of lower uptake of lycopene by cultured cells when compared to the uptake of β-carotene under comparable conditions has been previously described with mouse embryo fibroblasts (C3H/10T1/2) (Bertram *et al.*, 1991) and with KB-1 human oral tumor cells (Livny *et al.*, 2002), as models. Interestingly, in the study of Livny and associates, a two-day incubation of KB-1 cells with 7 μM β-carotene or lycopene in the medium resulted in an approximately 13 fold

greater accumulation of β-carotene when compared to that of lycopene (Livny *et al.*, 2002).

For many years, cell culture studies with carotenoids have been greatly hindered because of the extreme lipophilicity and instability of these compounds in oxygen containing atmospheres (Bertram, 1991; Britton, 1995; Martin *et al.*, 1997). Numerous methods have been proposed to overcome the problems related to the hydrophobicity of the carotenoids. For example, various types of liposomes have been employed, for delivery. However, rather low carotenoid cellular levels can be achieved with this method (Lowe *et al.*, 1999) and cytotoxic effects of some liposome constituents have been reported (IARC Working Group for the Evaluation of Cancer, 1998). Water-miscible beadlet formulations have also been used, but this method is limited in that the beadlet formulation contains many other chemical constituents (90%). A number of organic solvents (hexane, chloroform dimethylsulfoxide (DMSO) and acetone: DMSO mixtures) have also been used for the delivery of β-carotene to cells, however, different problems have been associated with each solvent. (Bertram *et al.*, 1991, 1999; Cooney *et al.*, 1993). Other methods for delivery of carotenoids to cultured cells include carotenoid -enriched bovine serum (Williams *et al.*, 2000) and nanoparticle formulations (Offord *et al.*, 2002). Tetrahydrofuran (THF) has been reported to be one of the best solvents for β-carotene (Khachik *et al.*, 1988) and to be effective in delivering carotenoids to cells with no apparent cytotoxicity and this method is being increasingly used since its introduction for this purpose (Cooney *et al.*, 1993).

The use of methyl-β–cyclodextrin (MβCD) has also recently been reported as a novel delivery system of carotenoids to cells in culture with high cellular carotenoid levels achieved. Human skin fibroblasts (HFP-1) accumulated 560-780 pmol/10^6cells after a 24hr incubation with 5 μM β-carotene and no isomerization was detectable in cells or medium (Pfitzner *et al.*, 2000). Overall, the delivery method might be of crucial importance not only because of the cellular level of β-carotene achieved and the isomerization allowed, but also because of possible interference of the vehicle employed with the measured biological end point.

In this context, it is interesting that our results contrast with those of a previous study published by Obermuller-Jevic and associates (1999), in which β-carotene at 0.5 and 5 μM concentrations in the medium enhanced the level of the HO-1 gene expression 1.3 and 2.7 fold, respectively, in normal human skin fibroblasts irradiated with a UVA dose of 200 kJ/m^2 over that observed with UVA alone.

A major difference may lie in the vehicle employed. Methyl-β–cyclodextrin (as used in the above mentioned study) is an efficient way of introducing carotenoids into cell culture (Pfitzner *et. al.*, 2000). However, cyclodextrins are cyclic polysaccharides with a hydrophobic cavity that extract cholesterol from lipid bilayers (Radhakrishnan and McConnell, 2000) and are frequently used agents for cholesterol depletion in studies of lipid raft disruption (Simons and Toomre, 2000). There is considerable evidence that extraction of cholesterol from membranes affects the rate of cholesterol biosynthesis in these cells (Radhakrishnan *et al*, 2000). Furthermore, β-Cyclodextrin leads to modulation of signalling pathways (Moran and Miceli, 1998; Roy *et. al.*, 1999; Gniadecki *et al.*, 2002). Therefore, it appears likely that methyl-β–cyclodextrin will complicate interpretation of experiments using additional modifying agents.

Although HO-1 appears to have a protective/anti-inflammatory role (see section 1.3), the induction of this enzyme is considered a marker of oxidative stress and, therefore, the suppression of this inducible response is considered as an indication of reduced oxidative stress. In this context it is noteworthy to mention that β-carotene and lycopene did not suppress the basal levels of HO-1 mRNA, as seen of the levels of sham irradiated FEK4 treated with the carotenoids. Interestingly, treatment of FEK4 cells with lycopene significantly induced basal levels of HO-1 mRNA **(Fig. 3.6.2-4)**.

Carotenoids are well known for their ability to quench singlet oxygen (see introduction). However, the use of β-carotene as a protective agent against oxidative damage in humans is currently under discussion, because of the findings of supplementation trials involving β-carotene (Omenn *et al.*, 1996; ATBC, 1994). These studies indicated that β-carotene supplementation is of little or no value in preventing cardiovascular disease, and the major cancers occurring in well nourished populations and may actually increase, rather than reduce, lung cancer incidence in asbestos exposed workers and

smokers. As a consequence of these findings, the trials of β-carotene have been terminated. It is consensus view, that more knowledge regarding transport, metabolism, tissue distribution and the molecular basis for the biological activities of carotenoids is needed in order to explain these unexpected findings and the mechanisms by which carotenoids might function in human tissues (Liu *et al*, 2000; Rock, 1997; Krinsky, 2001; Mayne, 1996; Omaye *et al.*, 1997; Palozza, 1998). Furthermore, for similar events to be avoided with lycopene, extensive studies are necessary (Clinton, 1998).

This is the first study to point to the possibility of an involvement of the PPAR/RXR heterodimer in mediating the biological effects of β-carotene in humans and may help explain the different outcome of β-carotene supplementation in asbestos exposed workers and smokers relative to the other groups, since both smoking and exposure to asbestos induce a high expression of cyclooxygenase 2 (COX-2) (Anto *et al.*, 2002; Zijlstra *et al.*, 1992; Schins and Donaldson, 2000). PPARs are currently being studied as targets for cancer prevention, but have also been shown to promote colon cancer in mice (reviewed in Kersten *et al*, 2000). Taking into account that the PPARs are thought to be major regulators of lipid absorption and metabolism (Kersten *et al*, 2000; Chawla *et al*, 2001) it is interesting that β-carotene has been shown to affect the transcription of key enzymes in cholesterol biosynthesis, i.e HMG Co A reductase (Fuhrman et al., 1997; Moreno *et al*, 1995).

4.1 Future research

For what concerns the modulation of UVA induced gene activation of HO-1 more experiments are needed in order to elucidate the underlying mechanism:

1. In order to examine whether the suppression of UVA induced HO-1 mRNA levels by β-carotene and lycopene is due to singlet oxygen quenching it would be of value

(i) to establish UVA dose response for the activation of HO-1 with or without β-carotene or lycopene (1, 2.5 and 5 μM) and an agent that enhances singlet oxygen lifetime (D_2O).

(ii) to establish the modulation of HO-1 mRNA levels in FEK4 cells exposed to singlet oxygen generated intracellularly from the thermal decomposition of an endoperoxide, such as 3,3'-(1,4-naphthylidene dipropionate ($DNPO_2$), with or without β-carotene or lycopene (1, 2.5 and 5 μM).

2. In order to investigate whether the suppression of HO-1 mRNA by carotenoids is accompanied by a suppression of protein levels and enzymatic activity, experiments need to be performed to establish the modulation of the UVA (100 and 250 kJ/m^2) induced HO-1 protein levels and activity in FEK4 cells by β-carotene or lycopene (1, 2.5 and 5 μM).

3. To further examine the hypothesis of COX-2 involvement in the observed suppression of UVA induction of HO-1 by carotenoids it would be important

(i) to establish the modulation of UVA induced COX-2 at the mRNA and protein level

(ii) to establish the activity of cox-2 as a function of concentration of β-carotene and lycopene and UVA dose applied to the cell cultures.

(iii) Establish the modulation of UVA induced HO-1 mRNA levels when the enzymatic activity of Cox-2 is selectively inhibited, by an agent that does not affect the constitutive form of the enzyme, such as celefoxib.

5 REFERENCES

1. Agulhon, H. (1912) *Annales de l' Institut Pasteur, Paris*, **26**, 38-47.

2. Ahmed, A.H., L.J. Notarianni, R.W. Jones (1995) A competitive single step reverse transcriptase-polymerase reaction assay for quantification of heme-oxygenase 1. *Analytical Biochemistry*, **237**, 149-152.

3. Allen, R.G. and M. Tresini (2000) Oxidative Stress and gene regulation. *Free Radical Biology and Medicine*, **28(3)**, 463-499.

4. Angel, P., A. Szabowski, M. Schorpp-Kistner (2001) Function and regulation of AP-1 subunits in skin physiology and pathology. *Oncogene*, **20**, 2413-2423.

5. Anto, R.J., A. Mukhopadhyay, S. Shishodia, C.G. Gairola, B.B. Aggarwal (2002) Cigarette smoke condensate activates nuclear transcription factor-kappa B through phosphorylation and degradation of I kappa B alpha: correlation with induction of cyclooxygenase-2. *Carcinogenesis*, **23 (9)**, 1511-1518.

6. Applegate, L.A, A. Noel, G. Vile, R.M. Tyrrell (1995) Two genes contribute to different extents to the HO enzyme activity measured in cultured human skin fibroblasts and keratinocytes: implications for protection against oxidant stress. *Photochemistry and Photobiology,* **61(3)**, 285-291.

7. Applegate, L.A, P. Luescher and R.M. Tyrrell (1991) Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells. *Cancer Research*, **51**, 974-978.

8. Applegate, L.A. and E. Frenk (1995) Cellular defense mechanisms of the skin against oxidative stress and in particular UVA radiation. *European Journal of Dermatology*, **5**, 97-103.

9. Arcari, P., R. Martinelli, F. Salvatore (1984) The complete sequence of a full-length cDNA for human-liver glyceraldehyde-3-phosphate dehydrogenase-evidence for multiple messenger-RNA species. *Nucleic Acids Research*, **12 (23)**, 9179-9189.

10. Armstrong, N.C., G. Papanga, E. Bunner, N.J. Miller, M. Shipley, C.A. Rice-Evans M.G. Marmot, and A.T. Dippock (1997) Reference values for a tocopherol and β-carotene in the Whitehall II Study. *Free Radical Research*, **27**, 207-219.

11. Aslund, F. and J. Beckwith (1999) Bridge over troubled waters: Sensing stress by disulfide bond formation. *Cell*, **96**, 751-753.

12. Bachelor, M.A., A.L. Silvers, G.T. Bowden (2002) The role of p38 in UVA-induced cyclooxygenase-2 expression in the human keratinocyte cell line, HaCaT. *Oncogene*, **21(46)**, 7092-7099.

13. Bachmann, H., A. Desbarats, P. Pattison, M. Sedgewick, G. Riss, A. Wyss, N. Cardinault, C. Duszka, R. Goralczyk and P. Grolier (2002) Feedback Regulation of ß,ß-Carotene 15,15'-Monooxygenase by Retinoic Acid in Rats and Chickens. *Journal of Nutrition*, **132**,3616-3622.

14. Bae, G.-U.,D.-W. Seo, H.-K. Kwon, H.Y. Lee, S. Hong, Z.-W. Lee, K.-S. Ha, H.-W. Lee and J.-W. Han (1999) Hydrogen peroxide activates p70 ^{S6k} signaling pathway. *Journal of Biological Chemistry*, **274 (46)**, 32596-32602.

15. Bae, Y.S., J.-Y. Sung, O.-S. Kim, Y.J. Kim, K.C. Hur, A. Kazlauskas, S.G. Rhee (2000) Platelet derived Growth Factor-induced H_2O_2 production requires the activation of Phosphatidylinositol 3-kinase. *Journal of Biological Chemistry*, **275(14)**, 10527-10531.

16. Bastie, J.-N., G. Deaspouy, N. Balitrand, C. Rochette-Egly, C. Chomienne, L. Delva (2001) The novel co-activator CRABPII binds to RARα and RXRα via two nuclear receptor interacting domains and does not require the AF-2 core. *FEBS Letters*, **507**, 67-73.

17. Basu -Modak, S., P. Luescher and R.M. Tyrrell (1996) Lipid metabolite involvement in the activation of the human HO-1 gene. *Free Radical Biology and Medicine*, **20(7)**, 887-897.

18. Basu-Modak, S. and R.M. Tyrrell (1993) Singlet oxygen. A primary effector in the UVA/near visible light induction of the human heme oxygenase gene. *Cancer Research*, **53**, 4505-4510.

19. Beato, M., P. Herrlich, G. Schutz (1995) Steroid-hormone receptors - many actors in search of a plot. *Cell*, **83(6)**, 851-857.

20. Ben-Dor, A., A. Nahum, M. Danilenko, Y. Giat, W. Stahl, H.D. Martin, T. Emmerich, N. Noy, J. Levy and Y. Sharoni (2001) Effects of acyclo-retinoic acid and lycopene on activation of the retinoic acid receptor and proliferation of mammary cancer cells. *Archives of Biochemistry and Biophysics*, **391(2)**, 295-302.

21. Bernard, P.S., R.S. Ajioka, J.P. Kushner, C.T. Wittwer (1998) Homogeneous Multiplex Genotyping of Hemochromatosis Mutations with Fluorescent hybridization Probes. *American Journal of Pathology*, **153**, 1055-1061.

22. Bertram, J.S. (1999) Carotenoids and gene regulation. *Nutrition Reviews*, **57(6)**, 182-191.

23. Bertram, J.S. and H. Bortkiewicz (1995) Dietary carotenoids inhibit Neoplastic transformation and modulate gene expression in mouse and Human Cells, *American Journal of Clinical Nutrition*, **62**(suppl), 1327S-36S.

24. Bertram, J.S., A. Pung, M. Churley, T.J. Kappock, L.R. Wilkins and R.V. Cooney. (1991) Diverse carotenoids protect against chemically induced neoplastic transformation. *Carcinogenesis*, **12(4)**, 671-678.

25. Block, G., B. Patterson, A. Subar (1992) Fruit, Vegetables, And Cancer Prevention - A Review of The Epidemiologic Evidence. *Nutrition And Cancer-An International Journal*, **18 (1)**, 1-29.

26. Blot, W.J., J.Y. Li, P.R. Taylor, W.D. Guo, S. Dawsey, G.Q. Wang, C.S. Yang, S.F. Zheng, M. Gail, G.Y. Li, Y. Yu, B.Q. Liu, J. Tangrea, Y.H. Sun, F.S. Liu, J.F. Fraumeni, Y.H. Zhang, B. Li (1993) Nutrition Intervention Trials In Linxian, China. -Supplementation with specific vitamin mineral combinations, cancer incidence, and disease-specific mortality in the general-population. *Journal of The National Cancer Institute*, **85 (18)**, 1483-1492.

27. Botros, F.T., M. Laniado-Schwartzman, N.G. Abraham (2002) Regulation of cyclooxygenase- and cytochrome P450-derived eicosanoids by heme oxygenase in the rat kidney. *Hypertension,* **39**, 639.

28. Brady, W.E., J.A. Mares-Perlman, P. Bowen, M. Stacewicz-Sapuntzakis (1996) Human serum carotenoid concentrations are related to physiologic and lifestyle factors. *Journal of Nutrition*, **126 (1)**, 129-137.

29. Britton, G. (1995) Structure and properties of carotenoids in relation to function. *FASEB Journal*, **9**, 1551-1558.

30. Briviba, K., K. Schnabele, E. Schwertle, M. Blockhaus, G. Rechkemmer (2001) beta-carotene inhibits growth of human colon carcinoma cells in vitro by induction of apoptosis. *Biological Chemistry*, **382 (12)**, 1663-1668.

31. Brown, D.A., E. London (2000) Structure and function of sphingolipid and cholesterol-rich membrane rafts. *Journal of Biological Chemistry,* **275 (23),** 17221-17224.

32. Brown, M.S. and J.L. Goldstein (1999) A proteolytic pathway that controls the cholesterol content of membranes, cells and blood *Proceedings National Academy of Science, USA*, **96,** 11041-11048.

33. Brown, M.S., J. Ye, R.B. Rawson and J. Goldstein (2000) Regulated Intramembrane Proteolysis: A control mechanism conserved from bacteria to humans. *Cell*, **100**, 391-398.

34. Brubacher, G.B., H. Weiser (1985) The vitamin A activity of beta-carotene. *International Journal of Vitamin and Nutrition Research*, **55(1)**, 5-15.

35. Burdon, R.H. (1995) Superoxide and hydrogen peroxide in relation to mammalian cell. *Free Radical Biology and Medicine*, **4**, 775-794.

36. Burton, G.W. and K.U. Ingold (1984) β-carotene: An unusual type of lipid antioxidant. *Science*, **224**, 569-573.

37. Cacciola, S.A., L.A. Cohen, K. Kashfi (1999) Lycopene inhibits proliferation and regulates cyclooxygenase-2 gene expression in neoplastic rat mammary epithelial cells. *FASEB Journal*, **13 (4)**, A551-A551 Part 1 Suppl. S.

38. Camhi, S.L., J. Alam, G.W. Wiegand, B.Y. Chin and A.M. Choi (1998) Transcriptional activation of the HO-1 gene by lipopolysaccharide is mediated by 5' distal enhancers: role of reactive oxygen intermediates and AP-1. *American Journal of Respiratory Cell and Molecular Biology*, **18 (2)**, 226-234.

39. Campbell, D.R., M.D. Gross, M.C. Martini, G.A. Grandits, J.L. Slavin, J.D. Potter (1994) Plasma carotenoids as biomarkers of vegetable and fruit intake. *Cancer Epidemiology Biomarkers and Prevention*, **3 (6)**, 493-500.

40. Carughi, A., F.G. Hooper (1994) Plasma carotenoid concentrations before and after supplementation with a carotenoid mixture. *American Journal of Clinical Nutrition,* **59 (4)**, 896-899.

41. Chamberlain, J., S.H. Moss (1987) Lipid-Peroxidation and other membrane damage produced in Escherichia *Coli* K1060 by Near-UV Radiation and Deuterium Oxide. *Photochemistry and Photobiology*, **45 (5)**, 625-630.

42. Chambon, P. (1996) A decade of molecular biology of retinoic acid receptors. *FASEB Journal*, **10**, 940-954.

43. Chawla, A., J.J. Repa, R.M. Evans and D. Mangelsdorf (2001) Nuclear receptors and lipid physiology: Opening the X files. *Science*, **294**, 1866-1870.

44. Chen, J.D., R.M. Evans (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature*, **377 (6548)**, 454-457.

45. Chinetti, G., J.C. Fruchart, B. Staels (2000) Peroxisome proliferator-activated receptors (PPARs): Nuclear receptors at the crossroads between lipid metabolism and inflammation. *Inflammation Research*, **49 (10)**, 497-505.

46. Chomczynski, P. and N. Sacchi (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, **162**, 156-159.

47. Christensen, R.L. (1999) Electronic states of Carotenoids Advances in Photosynthesis (Vol.8) the Photochemistry of Carotenoids. Edited by H.A. Frank, A.J. Young, G. Britton and R.J. Cogdell, **8**, 137-159.

48. Clevidence, B.A. and J.G. Bieri (1993) Association of carotenoids with human plasma lipoproteins. *Methods in Enzymology*, **214**, 33-46.

49. Clinton, S.K. (1998) Lycopene: Chemistry, Biology and Implications for Human health and Disease. *Nutrition Reviews*, **56(2)**, 35-51.

50. Clinton, S.K. (1999) The dietary antioxidant network and prostate carcinoma. *Cancer*, **86(9)**, 1629-1631.

51. Clinton, S.K., C. Emenhiser, S.J. Schwartz, D.G. Bostwick, A.W. Williams, B.J. Moore, J.W. Erdman (1996) cis-trans lycopene isomers, carotenoids, and retinol in the human prostate. *Cancer Epidemiology Biomarkers and Prevention*, **5 (10)**, 823-833.

52. Conn, P.F., W. Schalch, T.G. Truscott (1991) The singlet oxygen and carotenoid interaction. *Journal of Photochemistry and Photobiology B: Biology*, **11**, 41-47.

53. Connor, M.J., L.A. Wheeler (1987) Depletion of cutaneous glutathione by ultraviolet-radiation. *Photochemistry and Photobiology*, **46 (2)**, 239-245.

54. Cook, N.R., M.J. Stampfer, J. Ma, J.E. Manson, F.M. Sacks, J.E. Buring, C.H. Hennekens (2000) β-carotene supplementation for patients with low baseline levels and decreased risks of total and prostate carcinoma. *Cancer*, **86(9)**, 1783-1792.

55. Cooney, R.V., T.J. Kappock, A. Pung, J.S. Bertram (1993) Solubisation, cellular uptake and activity of β-carotene and other carotenoids as inhibitors of neoplastic transformation in cultured cells. *Methods in Enzymology*, **214**, 55-68.

56. Czech, M.P. (2000) PIP2 and PIP3: Complex Roles at the Cell Surface. *Cell*, **100**, 603-606.

57. Dalton, T.D., H.G. Shertzer and A. Puga (1999) Regulation of Gene Expression by Reactive Oxygen. *Annual Review of Pharmacology and Toxicology*, **39**, 67-101.

58. Dawson, M.I. (2000) The importance of vitamin A in nutrition. *Current Pharmaceutical Design*, **6 (3)**, 311-325.

59. De Pee, S., C.E. West, D. Permaesih, S. Martuti, Muhilal, J.G.A.J. Hautvast (1998) Orange fruit is more effective than are dark-green, leafy vegetables in increasing serum concentrations of retinol and beta-carotene in schoolchildren in Indonesia. *American Journal of Clinical Nutrition*, **68 (5)**, 1058-1067.

60. DeBruyne, L.A., J.C. Magee, R. Buelow and J.S. Bromberg (2000) Gene transfer of immunomodulatory peptides correlates with heme oxygenase-1 induction and enhanced allograft survival. *Transplantation*, **69**, 120–128.

61. Delva, L., J.-N. Bastie, C. Rochette-Egly, R. Kraiba, N. Balitrand, G. Despouy, P. Chambon and C. Chomienne (2001) Physical and functional interactions between Cellular retinoic acid binding Protein II and the Retinoic Acid Dependent Nuclear Complex. *Molecular and Cellular Biology*, **19(10)**, 7158-7167.

62. Dennery, P.A., G. Visner, Y-H. Weng, X. Nguyen, F. Lu, D. Zander and G.Yang (2003) Resistance to hyperoxia with heme-oxygenase-1 disruption: role of iron. *Free Radical Biology and Medicine*, **34**, 124-133.

63. Desvergne, B. and W. Wahli (1999) Peroxisome proliferator-activated receptors: Nuclear Control of metabolism. *Endocrine Reviews*, **20(5)**, 649-688.

64. Di Mascio, P., S. Kaiser, H. Sies (1989) Lycopene is the most efficient biological carotenoid singlet oxygen quencher. *Archives of Biochemistry and Biophysics*, **274(2)**, 532-538.

65. Duester, G. (2000) Families of retinoid dehydrogenases regulating vitamin A function. *European Journal of Biochemistry*, **267**, 4315-4324.

66. Edge, R. and T.G. Truscott (1999) In: Advances in Photosynthesis. The Photochemistry of Carotenoids. Edited by H.A. Frank, A.J. Young, G. Britton and R.J. Cogdell, **12**, 223-234.

67. Edwards, P.A., D. Tabor, H.R. Kast, A. Venkateswaran (2000) Regulation of gene expression by SREBP and SCAP. *Biochimica et Biophysica Acta*, **1529**, 103-113.

68. Eisenstark, A., G. Perrot (1987) Catalase has only a minor role in protection against near-ultraviolet radiation-damage in bacteria. *Molecular and General Genetics*, **207 (1)**, 68-72.

69. Epstein, J.H. (1977) Effects of β–carotene on Ultraviolet induced cancer formation in hairless mouse skin. *Photochemistry and Photobiology*, **25**, 211-213.

70. Erdman, J., T.L. Bierer and E.T. Gugger (1993) Absorption and transport of carotenoids. *Annals of New York Academy of Sciences,* **691**, 76-85.

71. Faure, H., V. Fayol, C. Galabert, P. Grolier, G Le Moel, J.P. Steghens, A. Van Kappel, F. Nabet (1999) Carotenoids: 1. Metabolism and Physiology. *Annales de Biologie Clinique*, **57(2)**, 169-83.

72. Feng, L., Y.Y. Xia, G.E. Garcia, D. Huang, C.B. Wilson (1995) Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor-necrosis-factor-alpha, and lipopolysaccharide. *Journal of Clinical Investigation*, **95 (4)**, 1669-1675.

73. Finsen, N.R. (1900) Neue Untersuchungen über die Entwirkung des Licthtes auf die Haut. *Mittelungen aus Finsens medizinishem Institut*, **1**, 8-34.

74. Foresti, R., J.E. Clark, C.J. Green, R. Motterlini (1997) Thiol compounds interact with nitric oxide in regulating heme oxygenase induction in endothelial cells. *Journal of Biological Chemistry*, **272(29)**, 18411-18417.

75. Fourtanier, A., A. Gueniche, D. Compan, S.L. Walker, A.R. Young (2000) Improved protection against solar-simulated radiation-induced immunosuppression by a sunscreen with enhanced ultraviolet a protection. *Journal of Investigative Dermatology*, **114 (4)**, 620-627.

76. Frederick, J.E. and D. Lubin (1988) Possible long-term changes in biologically active ultraviolet radiation reaching the ground. *Photochemistry and Photobiology*, **47**, 571-578.

77. Freeman, W.M., S.J. Walker and K.E. Vrana (1999) Quantitative RT-PCR: Pitfalls and Potential. *BioTechniques*, **26**, 112-125.

78. Frommel, T.O., S. Mobarhan, M. Doria, A.G. Halline, G.D. Luk, P.E. Bowen, A. Candel, Y.L. Liao (1995) Effect of beta-carotene supplementation on indexes of colonic cell-proliferation. *Journal of The National Cancer Institute*, **87 (23)**, 1781-1787.

79. Fuhrman, B., A. Elis, M. Aviram (1997) Hypocholesterolemic effect of lycopene and beta-carotene is related to suppression of cholesterol synthesis and augmentation of LDL receptor activity in macrophages. *Biochemical and Biophysical Research Communications*, **233 (3)**, 658-662.

80. Fuller, C.J., H. Faulkner, A. Bendich, R.S. Parker, D.A. Roe (1992) Effect of beta-carotene supplementation on photosuppression of delayed-type hypersensitivity in normal young men. *American Journal of Clinical Nutrition*, **56 (4)**, 684-690.

81. Furuchi, T. and R.G.W. Anderson (1998) Cholesterol depletion of caveolae causes hyperactivation of extracellular signal related kinases (ERK). *Journal of Biological Chemistry*, **273 (83)**, 21099-21104.

82. Gaboriau, F., P. Morliere, I. Marquis, A. Maysan, M. Geze, L. Dubertet J. (1993) Membrane damage induced in cultured human skin fibroblasts by UVA irradiation. *Photochemistry and Photobiology*, **58**, 515-520.

83. Galbraith, R. (1999) Heme oxygenase: Who needs it? *Proceedings of the Society for Experimental Biology and Medicine*, **222**, 299-305.

84. Garavelli, M., F. Bernardi, M. Olivucci, M.A. Robb (1998) DFT study of the reactions between Singlet Oxygen and a Carotenoid model. *Journal of the American Chemical Society*, **120**, 10210-10222.

85. Gerster, H. (1997) The potential role of lycopene for human health. *Journal of the American College of Nutrition*, **16 (2)**, 109-126.

86. Ghyselinck, N.B., C. Bavik, V. Sapin, M. Mark, D. Bonnier, C. Hindelang, A. Dierich, C.B. Nilsson, H. Hakansson, P. Sauvant, V. Azais-Braesco, M. Frasson,

S. Picaud, P. Chambon (1999) Cellular retinol-binding protein I is essential for vitamin A homeostasis. *EMBO Journal,* **18 (18)**, 4903-4914.

87. Giguere, V. (1999) The orphan receptors: From gene to function. *Endocrine Reviews*, **20 (5)**, 689-725.

88. Giovannucci, E. (1999) Tomatoes, tomato-based products, lycopene, and cancer: Review of the Epidemiologic literature. *Journal of the National Cancer Institute*, **91**, 317–31.

89. Giovannucci, E.L., A. Ascherio, E.B. Rimm, M.J. Stampfer, G.A. Colditz, W.C. Willett (1995) Intake of carotenoids and retinol in relationship to risk of prostate cancer. *Journal of the National Cancer Institute,* **87**, 1767–76.

90. Gniadecki, R., N. Christofersen, H.C. Wolf (2002) Cholesterol rich plasma membrane domains in keratinocytes: Importance in the baseline and UVA induced generation of reactive oxygen species (ROS). *Journal of Investigative Dermatology*, **118**, 582-588.

91. Gonzalez, J.M., C. Saiz-Jimenez (2002) A fluorimetric method for the estimation of G+C mol% content in microorganisms by thermal denaturation temperature. *Environmental Microbiology*, **4 (11)**, 770-773.

92. Goodman, R.H. and S. Smolik (2000) CBP/p300 in cell growth, transformation, and development. *Genes and Development*, **14**, 1553-1557.

93. Greenberg, E.R., J.A. Baron, T.A. Stukel, M.M. Stevens, J.S. Mandel, S.K. Spencer, P.M. Elias, N. Lowe, D.W. Nierenberg, G. Bayard, J.C. Vance, D.H. Freeman, W.E. Clendenning, T. Kwan and The Skin Cancer Prevention Study Group (1990) A clinical trial of β-carotene to prevent basal-cell and squamous-cell cancers of the skin. *New England Journal of Medicine*, **323(12)**, 789-795.

94. Greenberg, E.R., J.A. Baron, T.B. Tosteson, D.H. Freeman, G.J. Beck, J.H. Bond, T.A. Collacchio, H.D. Frankl, R.W. Haile, J.S. Mandel, D.W. Nierenberg, R. Rothstein, D.C. Snover, M.M. Stevens, R.W. Summers, R.U. Van Stolk and The Polyp Prevention Study Group (1994) A clinical trial of antioxidant Vitamins to prevent colorectal adenoma. *New England Journal of Medicine*, **331**, 141-147.

95. Grolier, P, V. Azaisbraesco, L. Zelmire, H. Fessi (1992) Incorporation of carotenoids in aqueous systems - uptake by cultured rat hepatocytes. *Biochimica et Biophysica Acta*, **1111 (1)**, 135-138.

96. Gruszecki, W.I. (1999) Carotenoids in Membranes. Advances in Photosynthesis (Vol.8) the Photochemistry of Carotenoids, **8**, 363-379. Edited by H.A. Frank, A.J. Young, G. Britton and R.J. Cogdell.

97. Gupta, S. and H. Mukhtar (2002) Chemoprevention of skin cancer: Current status and future prospects. *Cancer and Metastasis Reviews*, **21**, 363-380.

98. Haider, A., R. Olszanecki, R. Gryglewski, M. L. Schwartzman, E. Lianos, A. Kappas, A. Nasjletti and N.G. Abraham (2002) Regulation of cyclooxygenase by the heme-heme oxygenase system in microvessel endothelial cells. *Pharmacology and Experimental Therapeutics*, **300 (1)**, 188-194.

99. Halliwell, B and J. Gutteridge (1999) in *Free Radicals in Biology and Medicine, 3rd Edition* (Oxford Science Publications, UK, 1999).

100. Hanson, D.L. and V.A. DeLeo (1989) Long wave ultraviolet light stimulates arachidonic acid release and cyclooxygenase activation in mammalian cells in culture. *Photochemistry and Photobiology*, **49 (4)**, 423-430.

101. Hanson, D.L. and V.A. DeLeo (1990) Long wave ultraviolet light induces phospholipase activation in cultured human epidermal keratinocytes. *Journal of Investigative Dermatology*, **49**, 158-163.

102. Harris, S., D.B. Jones (1997) Optimisation of the polymerase chain reaction. *British Journal of Biomedical Science*, **54 (3)**, 166-173.

103. Harrison, G.I. and A.R. Young (2002) Ultraviolet radiation –induced erythema in human skin. *Methods*, **28**, 14-19.

104. Hebuterne, X., X.-D. Wang, E.J. Johnson, N.I Krinsky, R.M. Russell (1995) Intestinal absorption and metabolism of 9-cis-β-carotene in vivo: Biosynthesis of 9-cis-retinoic acid. *Journal of Lipid Research,* **36**, 1264-1273.

105. Heid, C.A., J. Stevens, K.J. Livak, P.M. Williams (1996) Real time quantitative PCR. *Genome Research*, **6 (10)**, 986-994.

106. Heinonen, O.P. and D. Albunen (1994) The effect of Vitamin E and β-carotene on the incidence of lung cancer and other cancers in male smokers. *The New England Journal of Medicine*, **330**, 1029-1035.

107. Hennekens, C.H., J.E. Buring, J.E. Manson, M. Stampfer, B. Rosner, N.R. Cook, C. Belanger, F. LaMotte, M. Gaziano, P. Ridker, W. Willett, R. Peto (1996)

Lack of effect of long-term supplementation with β-carotene on the incidence of malignant neoplasms and cardiovascular disease (Physicians Health Study). *New England Journal of Medicine*, **334(18)**, 1145-49.

108. Herraiz, L.A., W.C. Hsieh, R.S. Parker, J.E. Swanson, A. Bendich, D.A. Roe (1998) Effect of UV exposure and beta-carotene supplementation on delayed-type hypersensitivity response in healthy older men. *Journal of the American College of Nutrition*, **17 (6)**, 617-624.

109. Herrlich, P. and F.D. Boehmer (2000) Redox Regulation of Signal Transduction in Mammalian cells. *Biochemical Pharmacology*, **59**, 35– 41.

110. Heseker, H., M. Kohlmeier, R. Schneider, A. Speitling, W. Kubler (1991) Vitamin-versorgung Erwachsener in der Bundesrepublik Deutschland. *Ernahrungs Umschau*, **38**, 227-233.

111. Heyman, R.A., D.J Mangelsdorf, J.A. Dyck, R.B. Stein, G. Eichele, R.M. Evans and C. Thaller (1992) 9-cis Retinoic Acid is a High Affinity Ligand for the Retinoid X Receptor. *Cell*, **68**, 397-406.

112. Higuchi, R., C. Fockler, G. Dollinger and R. Watson (1993) Kinetic PCR Analysis: Real Time Monitoring of DNA Amplification Reactions. *Biotechnology*, **11**, 1026-1030.

113. Holland, P.M., R.D. Abraamson, R. Watson and D.H. Gelfand (1991) Detection of specific polymerase chain reaction product by utilising the 5'→3' exonuclease activity of *Thermus aquaticus. Proceedings of the National Academy of Science, USA*, **88**, 7276-7280.

114. IARC Working Group for the Evaluation of Cancer (1998) In: IARC Handbooks of Cancer Prevention, Volume 2, Carotenoids. Published by the International Agency for Research on Cancer, World Health Organization, Lyon, France.

115. Iftikhar, S., H. Lietz, S. Mobarhan and T.O. Frommel (1996) In vitro β–carotene toxicity for Human colon cancer cells. *Nutrition and Cancer,* **25**, 221-230.

116. Immenschuh, S., G. Ramadori (2000) Gene regulation of Heme oxygenase-1 as a therapeutic target. *Biochemical Pharmacology*, **60**, 1121-1128.

117. Irani, K., Y. Xia, J.L. Zweier, S.J. Sollott, C.J. Der, E.R. Fearon, M. Sundaresan, T. Finkel, P.J. Goldschmidt-Clermont (1997) Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. *Science*, **275**, 1649-1652.

118. Jones, S.A., J.D. Wood, M.S. Coffey, and O.T.G. Jones (1994) The functional Expression of p47-phox and p67-phox may contribute to the generation of superoxide by an NADPH oxidase-like system in human fibroblasts. *FEBS Letters*, **355**, 178-182.

119. Jornot, L. and A.F. Junot (1993) Variable glutathione levels and expression of antioxidant enzymes in human endothelial cells. *American Journal of Physiology*, **264**, L482-L489.

120. Kanetsky, P.A., M.D. Gammon, J. Mandelblatt, Z.F. Zhang, E. Ramsey, A. Dnistrian, E.P. Norkus, T.C. Wright (1998) Dietary intake and blood levels of lycopene: Association with cervical dysplasia among non-Hispanic, black women. *Nutrition and Cancer-An International Journal,* **31 (1)**, 31-40.

121. Kaplan, L.A., J.M. Lau, E.A. Stein (1990) Carotenoid composition, concentrations, and relationships in various human organs. *Clinical Physiology and Biochemistry*, **8 (1)**, 1-10.

122. Karas, M., H. Amir, D. Fishman, M. Danilenko, S. Segal, A. Nahum, A. Koifmann, Y. Giat, J. Levy, Y. Sharoni (2000) Lycopene interferes with cell cycle progression and insulin-like growth factor I signalling in mammary cancer cells. *Nutrition and Cancer-An International Journal*, **36 (1)**, 101-111.

123. Kastner, P., M. Mark, N. Ghyselinck, W. Krezel, V. Dupe, J.M. Grondona and P. Chambon (1997) Genetic evidence that the retinoid signal is transduced by heterodimeric RXR/RAR functional units during mouse development. *Development*, **124**, 313-326.

124. Keen, J.N., I. Caceres, E.E. Eliopoulos, P.F. Zagalsky, J.B.C. Findlay (1991) Complete sequence and model for the C1 subunit of the carotenoprotein, Crustacyanin, and model for the dimer, beta-crustacyanin, formed from the C1 and A2 subunits with astaxanthin. *European Journal of Biochemistry*, **202 (1)**, 31-40.

125. Kennedy, A.R., N.I. Krinsky (1994) Effects of retinoids, beta-carotene, and canthaxanthin on UV-induced and X-ray-induced transformation of C3H10T1/2 cells in-Vitro. *Nutrition and Cancer-An International Journal*, **22 (3)**, 219-232.

126. Kermekchiev, M., M. Muramatsu (1993) Presence of an inhibitor of RNA polymerase-I mediated transcription in extracts from growth arrested mouse cells. *Nucleic Acids Research*, **21(3)**, 447-453.

127. Kersten, S., B. Desvergne and W. Wahli (2000) Roles of PPARs in health and disease. *Nature*, **405**, 421-424.

128. Keyse, S.M. (1995) An emerging family of dual specificity MAP-kinase phosphatases. *Biochimica et Biophysica Acta*, **1265**, 152-160.

129. Keyse, S.M. and R.M. Tyrrell (1989) Heme Oxygenase is the major 32kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide and sodium arsenite. *Proceedings of the National Academy of Sciences, USA*, **86**, 99-103.

130. Keyse, S.M., L.A. Applegate, Y. Tromvoukis and R.M.Tyrrell (1990) Oxidant stress leads to transcriptional activation of the human HO gene in cultured skin fibroblasts. *Molecular and Cell Biology*, **10(9)**, 4967-69.

131. Khachik, F., G.R. Beecher, J.T. Vanderslice, G. Furrow (1988) Liquid chromatographic artefacts and peak distortion: Sample- solvent interactions in the separation of carotenoids. *Analytical Chemistry*, **60 (8)**, 807-811.

132. Kiefer, C., S. Hessel, J.M. Lampert, K. Vogt, M.O. Lederer, D.E. Breithaupt, J. von Lintig (2001) Identification and characterisation of a mammalian enzyme catalysing the assymetric oxidative cleavage of provitamin A. *Journal of Biological Chemistry*, **276(17)**, 14110-14116.

133. Kim, S.J., E. Nara, H. Kobayashi, J. Terao, A. Nagao (2001) Formation of cleavage products by autoxidation of lycopene. *Lipids*, **36 (2)**, 191-199.

134. Kliewer, S.A., J.M. Lehmann, T.M. Willson (1999) Orphan Nuclear Receptors: Shifting endocrinology into reverse. *Science*, **284**, 757-760.

135. Klotz, L.-O., C. Pellieux, K. Briviba, C. Pierlot, J.-M. Aubry and H. Sies (1999) Mitogen-activated protein kinase (p38-, JNK-, ERK-) activation pattern induced by extracellular and intracellular singlet oxygen and UVA. *European Journal of Biochemistry*, **260 (3)**, 917-922.

136. Klotz, L-O, K. Briviba, H. Sies (1997) Singlet oxygen mediates the activation of JNK by UVA radiation in human skin fibroblasts. *FEBS Letters*, **379**, 289-291.

137. Knebel, A., H.J. Rahmsdorf, A. Ulrich, P. Herrlich (1996) Dephosphorylation of receptor tyrosine kinases as target of regulation by radiation, oxidants, or alkylating agents. *EMBO Journal*, **15 (19)**, 5314- 5325.

138. Krinsky, N.I (1994) The biological properties of carotenoids. *Pure and Applied Chemistry*, **66**, 1003-1010.

139. Krinsky, N.I. (1993) Actions of carotenoids in Biological Systems. *Annual Review of Nutrition*, **13**, 561-587.

140. Krinsky, N.I. (2001) Carotenoids as antioxidants. *Nutrition*, **17 (10)**, 518-817.

141. Krutmann, J., M. Grewe (1995) Involvement of cytokines, DNA-Damage, and reactive oxygen intermediates in radiation-induced modulation of intercellular-adhesion molecule-1 expression. *Journal of Investigative Dermatology*, **105 (1)**, S67-S70 Suppl.

142. Kulig, M. and M. Smith (1973) Sterol metabolism XXV: Cholesterol oxidation by singlet molecular oxygen. *Journal of Organic Chemistry,* **38**, 3639-3652.

143. Kvam, E., A. Noel, S. Basu-Modak and R.M. Tyrrell (1999) Cyclooxygenase dependent release of heme from microsomal hemeproteins correlates with induction of heme oxygenase 1 transcription in human fibroblasts. *Free Radical Biology Medicine*, **26 (5-6)**, 511-517.

144. Kvam, E., R.M. Tyrrell (1997) Induction of oxidative DNA base damage in human skin cells by UV and near visible radiation. *Carcinogenesis*, **18 (12)**, 2379-2384.

145. Lamb, N.J., G.J. Quinlan, S. Mumby, T.W. Evans, J.M.C. Gutteridge (1999) Haem oxygenase shows pro-oxidant activity in microsomal and cellular systems: implications for the release of low-molecular-mass iron. *Biochemical Journal*, **344**, 153-158.

146. Laniado-Schwartzman, M., N.G. Abraham, M. Conners, M.W. Dunn, R.D. Levere and A. Kappas (1997) Heme oxygenase induction with attenuation of experimentally induced corneal inflammation. *Biochemical Pharmacology*, **53**, 1069–1075.

147. Lautier, D., P. Luscher, R.M. Tyrrell (1992) Endogenous Glutathione Levels Modulate Both Constitutive and UVA Radiation Hydrogen-Peroxide Inducible Expression of the Human Heme Oxygenase Gene. *Carcinogenesis*, **13 (2)**, 227-232.

148. Leblanc, B.P., H.G. Stunnenberg (1995) 9-cis-retinoic acid signaling - changing partners causes some excitement. *Genes and Development*, **9 (15)**, 1811-1816.

149. Lee, H.-Y., Y.-A. Suh, M.J. Robinson, J.L. Clifford, W.H. Hong, J.R. Woodgett, M.H. Cobb, D.J. Mangelsdorf, J.M. Kurie (2000) Stress pathway activation

induces phosphorylation of Retinoid X Receptor. *Journal of Biological Chemistry*, **275(41)**, 32193-32199.

150. Levine, R.L., J.A. Williams, E.R. Stadtman, E. Shacter (1994) Carbonyl assays for determination of oxidatively modified proteins. *Methods In Enzymology*, **233**, 346-357.

151. Levy, J., E. Bosin, B. Feldman, Y. Giat, A. Miinester, M. Danilenko, Y. Sharoni (1995) Lycopene is a more potent inhibitor of human Cancer Cell proliferation than either alpha-carotene or beta-carotene. *Nutrition and Cancer-An International Journal*, **24 (3)**, 257-266.

152. Lie, Y.S and C.J. Petropoulos (1998) Advances in quantitative PCR technology: 5' nuclease assays. *Current opinion in Biotechnology*, **9**, 43-48.

153. Liebler, D.C. (1993) Antioxidant action actions of carotenoids. *Annals of the New York Academy of Sciences*, **669**, 20-31.

154. Liu, C., X.D. Wang, R.T. Bronson, D.E. Smith, N.I. Krinsky, R.M. Russell (2000) Effects of physiological versus pharmacological beta-carotene supplementation on cell proliferation and histopathological changes in the lungs of cigarette smoke-exposed ferrets. *Carcinogenesis*, **21 (12)**, 2245-2253.

155. Livny, O., I. Kaplan, R. Reifen, S. Polak-Charcon, Z. Madar and B. Schwartz (2002) Lycopene Inhibits Proliferation and Enhances Gap-Junction Communication of KB-1 Human Oral Tumor Cells. *Journal of Nutrition*, **132**, 3754-3759.

156. Lowe, L.A., Booth, A.J. Young, R.F. Bilton (1999) Lycopene and beta carotene protect against oxidative damage in HT29 cells at Low concentrations but rapidly lose this capacity at higher Doses. *Free Radical Research*, **30,** 141-151.

157. Lu, Q.-Y., J.-C. Hung, D. Heber, V. Go, V. E. Reuter, C. Cordon-Cardo, H. I. Scher, J. R. M. and Z.-F. Zhang (2001) Inverse Associations Between Plasma Lycopene and Other Carotenoids and Prostate Cancer. *Cancer Epidemiology Biomarkers & Prevention*, **10**, 749-756.

158. Maines, M.D. (1996) New developments in the in the regulation of heme metabolism and their implications. *Critical Reviews in Toxicology*, **12**, 241-314.

159. Maines, M.D. (1997) The Heme Oxygenase System: A Regulator of Second Messenger Gases. *Annual Review of Pharmacology and Toxicology*, **37**, 517-554.

160. Mangelsdorf, D.J. and R. Evans (1995) The RXR Heterodimers and Orphan Receptors. *Cell*, **83**, 835-839.

161. Martin, G., H. Poirier, N. Hennuyer, D. Crombie, J.C. Fruchart, R.A. Heyman, P. Besnard, J. Auwerx (2000) Induction of the fatty acid transport protein 1 and acyl-CoA synthase genes by dimer-selective rexinoids suggests that the peroxisome proliferator-activated receptor-retinoid X receptor heterodimer is their molecular target. *Journal of Biological Chemistry*, **275 (17)**, 12612-12618.

162. Martin, K.R., G. Loo, M.L. Failla (1997) Human Lipoproteins as a Vehicle for the Delivery of Betacarotene and α-Tocopherol to HepG2 Cells. *Proceedings of the Society for Experimental Biology and Medicine*, **214**, 367-373.

163. Mathews-Roth, M. M. (1993) Carotenoids in erythropoietic protoporphyria and other photosensitivity diseases. *Annals of the New York Academy of Sciences*, **691**, 127-138.

164. Mayne, S.T. (1996) Beta carotene, carotenoids and disease prevention in humans. *FASEB Journal,* **10**, 690-701.

165. McCourbey, W.K Jr, T. J. Huang and M.D. Maines (1997) Isolation and characterisation of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *European Journal of Biochemistry*, **247**, 725-732.

166. Meister, A. and M.E. Andersen (1983) Glutathione. *Annual Review of Biochemistry*, **52**, 711-760.

167. Merkel, P.B. and D.R. Kearns (1972) Radiationless decay of Singlet Molecular Oxygen in Solution. An experimental and theoretical study of electronic-to-vibrational energy transfer. *Journal of the American Chemical Society*, **94(21)**, 7244- 7253.

168. Micozzi, M.S., E.D. Brown, B.K. Edwards, J.G. Bieri, P.R. Taylor, F. Khachik, G.R. Beecher, J.C. Smith (1992) Plasma carotenoid response to chronic intake of selected foods and beta-carotene supplements in men. *American Journal of Clinical Nutrition*, **55 (6)**, 1120-1125.

169. Mitchell, J.A., T.D. Warner (1999) Cyclo-oxygenase-2: pharmacology, physiology, biochemistry and relevance to NSAID therapy. *British Journal of Pharmacology*, **128 (6)**, 1121-1132.

170. Mitchell, R.L. and I.C. Andersen (1965) Catalase photoinactivation. *Science*, **150**, 74-76.

171. Moran, M. and M. Miceli (1998) Engagement of GPI-linked CD48 contributes to TCR signals and cytoskeletal reorganisation: a role of lipid rafts in T cell activation. *Immunity*, **9**, 787-796.

172. Moreno, F.S., M.R. Rosiello, S. Manjeshwar, R. Nath, P.M. Rao, S. Rajalakshmi, D.S.R. Sarma (1995) Effect of β-carotene on the expression of 3-hydroxy-methylglutaryl coenzyme A reductase in rat liver. *Cancer Letters*, **96**, 201-208.

173. Nagao, A., J.A. Olson (1994) Enzymatic formation of 9-cis, 13-cis, and all-trans retinals from isomers of β-carotene. *FASEB Journal*, **8**, 968-973.

174. Nagy, L., H.-Y. Kao, D. Chakravarti, R.J. Lin, C.A. Hassig, D.E. Ayer, S.L. Schreiber, and R.M. Evans (1997) Nuclear receptor Repression Mediated by a complex Containing SMRT, mSin3A and Histone Deacetylase. *Cell*, **89**, 373-380.

175. Nakano, H., F. Gasparro, J. Uitto (2001) UVA-340 as an energy source, mimicking natural sunlight, activates the transcription factor AP-1 in cultured fibroblasts: Evidence for involvement of protein kinase-C. *Journal of Investigative Dermatology*, **117 (2)**, 652.

176. Nara, E., H. Hayashi, M. Kotake, K. Miyashita, A. Nagao (2001) Acyclic carotenoids and their oxidation mixtures inhibit the growth of HL-60 human promyelocytic leukaemia cells. *Nutrition and Cancer-An International Journal*, **39 (2)**, 273-283.

177. Natarajan, V., W.M. Scribner, M.M. Taher (1993) 4-Hydroxynonenal, a metabolite of lipid-peroxidation, activates phospholipase-D in vascular endothelial-cells. *Free Radical Biology And Medicine*, **15 (4)**, 365-375.

178. Nierenberg, D.W., S.L. Nann (1992) A Method for determining concentrations of retinol, tocopherol, and 5 Carotenoids in human plasma and tissue samples. *American Journal of Clinical Nutrition*, **56 (2)**, 417-426.

179. Nierenberg, D.W., Y.M. Peng and D. Alberts (1989) Methods for determination of retinoids, a-tocopherols and carotenoids in human serum, plasma and other tissues. In: Nutrition and Cancer prevention, pp: 181-209, Moon, T.E. and Micozzi M.S. (eds) Markel Dekker, New York.

180. Nikawa, T., W.A. Schulz, C.E. van de Brink, M. Hanusch, P. van der Saag, and H. Sies. (1995) Efficacy of All-trans β-Carotene, Canthaxanthin, and All-trans-, 9-cis and 4-oxoretinoic acids in inducing differentiation of an F9 embryonal carcinoma RARβ-lacZ reporter cell line. *Archives of Biochemistry and Biophysics*, **316(2)**, 665-672.

181. Niki, E., Y. Yamamoto, E. Komuro, K. Sato (1991) Membrane damage due to lipid oxidation. *Journal of American Clinical Nutrition*, **53**, 201S-205S.

182. Noel, Al. and R.M. Tyrrell (1997) Development of refractoriness of induced human expression to reinduction by UVA irradiation and hemin. *Photochemistry and Photobiology*, **66(4)**, 456-463.

183. Nutter, L.M., E.E. Sierra, E.O. Ngo (1994) Heme oxygenase does not protect human-cells against oxidant stress. *Journal of Laboratory and Clinical Medicine*, **123 (4)**, 506-514.

184. Obermueller-Jevic, U.C., B. Schegel, A. Flaccus, H.K. Biesalski (2001) The effect of β-carotene on the expression of interleukin -6 and heme oxygenase -1 in UV irradiated human skin fibroblasts. *FEBS Letters*, **509**, 186-190.

185. Obermüller-Jevic, U.C., Francz, P.I, Frank, J., Flaccus, A. and H.K. Biesalski (1999) Enhancement of the UVA induction of haem oxygenase-1 expression by ß-carotene in human skin fibroblasts. *FEBS Letters*, **460**, 212-216.

186. Offord, E.A., J.C. Gautier, O. Avanti, C. Scaletta, F. Runge, K. Kraemer and L.A. Applegate (2002) Photoprotective Potential of Lycopene, β-Carotene, Vitamin E, Vitamin C and Carnosic Acid in UVA-Irradiated Human Skin Fibroblasts. *Free Radical Biology and Medicine*, **32(12)**, 1293-1303.

187. Okai, Y., K. Higashi-Okai (1996) Possible immunomodulating activities of carotenoids in *in vitro* cell culture experiments. *International Journal of Immunopharmacology*, **18(12)**, 753-758.

188. Oleastro, M., A. Menard, A. Santos, H. Lamouliatte, L. Monteiro, P. Barthelemy, F. Megraud (2003) Real-time PCR assay for rapid and accurate detection of point mutations conferring resistance to clarithromycin in *Helicobacter pylori*. *Journal of Clinical Microbiology*, **41 (1)**, 397-402.

189. Olson, J.A. (1994a) Hypovitaminosis A: contemporary scientific issues. *Journal of Nutrition*, **124(8 Suppl)**, 1461S-1466S.

190. Olson, J.A. (1994b) Absorption transport and metabolism of carotenoids in humans. *Pure and applied Chemistry*, **66**, 1011-1016.

191. Omaye, S.T., N.I. Krinsky, V.E. Kagan, S.T. Mayne, D.C. Liebler, W.R. Bidlack (1997) Beta-carotene: Friend or foe? *Fundamental and Applied Toxicology*, **40 (2)**, 163-174.

192. Omenn, G.S., E.G. Goodman, M.D. Thornquist, J. Balmes, M.R. Cullen, A. Glass, J.P. Keogh, F.L. Meyskens, B. Valamis, J.H. Williams, S. Barnhart, S. Hammer (1996) Effects of a combination of β-carotene and VitE on lung cancer and cardiovascular disease (CARET). *New England Journal of Medicine*, **334(18)**, 1150-55.

193. Oosthuizen, M.M.J., D. Greyling (1999) Antioxidants suitable for use with chemiluminescence to identify oxyradical species. *Redox Report*, **4 (6)**, 277-290.

194. Orlando, C., P. Pinzani and M. Pazzagli (1998) Developments in quantitative PCR. *Clinical Chemistry and Laboratory Medicine*, **36(5)**, 255-269.

195. Ortiz de Montelano, P.R. (2000) The mechanism of heme oxygenase. *Current Opinion in Chemical Biology*, **4**, 221-227.

196. Osada, K., A. Sevanian (2000) Cholesterol photodynamic oxidation by ultraviolet radiation and cholesterol ozonisation by ozone exposure. *Methods in Enzymology*, **319**, 188-196.

197. Ossola, J.O. and M.L. Tomaro (1998) Heme oxygenase induction by UVA radiation. A response to oxidative stress in rat liver. *The International Journal of Biochemistry and Cell Biology*, **30**, 285-292.

198. Otterbein, L.E. and A.M.K. Choi (2000) Heme oxygenase: colors of defense against cellular stress. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, **279**, L1029-L1037.

199. Otterbein, L.E., J.K. Kolls, L.L. Mantell, J.L. Cook, J. Alam and A.M. Choi (1999) Exogenous administration of heme oxygenase-1 by gene transfer provides protection against hyperoxia-induced lung injury. *Journal of Clinical Investigation*, **103**,1047–1054.

200. Palozza, P. (1998) Prooxidant Actions of Carotenoids in Biologic systems. *Nutrition Reviews*, **56(9)**, 257-265.

201. Palozza, P. and N. Krinsky (1992b) Beta carotene and α-tocopherol are synergistic antioxidants. *Archives of Biochemistry and Biophysics*, **297(1)**, 184-187.

202. Palozza, P. and N.I. Krinsky (1992a) Antioxidant Effects of Carotenoids *in Vivo* and *in Vitro*: An Overview. *Methods in Enzymology*, **213**, 403-420.

203. Palozza, P., C. Luberto, P. Ricci, E. Sgarlata, G. Calviello, G.M. Bartoli (1996) Effect of beta-carotene and canthaxanthin on tert-butyl hydroperoxide-induced lipid peroxidation in murine normal and tumor thymocytes. *Archives of Biochemistry and Biophysics*, **325 (2)**, 145-151.

204. Palozza, P., G. Calviello, S. Serini, N. Maggiano, P. Lanza, F.O. Raneletti and G.M. Bartoli (2001) β-carotene at high concentrations induces apoptosis by enhancing oxy-radical production in human adenocarcinoma cells. *Free Radical Biology and Medicine*, **30(9)**, 1000-1007.

205. Panahian, N., M. Yoshiura and M.D. Maines (1999) Overexpression of heme oxygenase-1 is neuroprotective in a model of permanent middle cerebral artery occlusion in transgenic mice. *Journal of Neurochemistry*, **72**, 1187–1203.

206. Parker, R.S. (1996) Absorption metabolism and transport of carotenoids. *FASEB Journal*, **10**, 542-551.

207. Parrish, T.A., T.M. Fitzpatrick, L. Tannenbaum, M.A. Tathak (1974) Photochemoterapy of psoriasis with oral methoxsalen and long wave ultraviolet light. *New England Journal of Medicine*, **291**, 1207-11.

208. Paton, M.G., S.H.P.P. Karunaratne, E. Yiakoumaki, N. Roberts, J. Hemingway (2000) Quantitative analysis of gene amplification in insecticide-resistant culex mosquitoes. *Biochemical Journal*, **346**, 17-24.

209. Peak, M.J., J.G. Peak (1991) Comparison of initial yields of DNA-to-protein crosslinks and single-strand breaks induced in cultured human cells by far-ultraviolet and near-ultraviolet light and X-rays. *Mutation Research*, **246**, 187-191.

210. Peak, M.J., J.G. Peak, B.A. Carnes (1987) Induction of direct and indirect single-strand breaks in human cell-DNA by far-ultraviolet and near-ultraviolet radiations - action spectrum and mechanisms. *Photochemistry and Photobiology*, **45 (3)**, 381-387.

211. Peng, Y.M., Y.S. Peng, Y. Lin, T. Moon, D.J. Roe, C. Rittenbaugh (1995) Concentration of plasma tissue diet relationships of carotenoids, retinoids and tocopherols in humans. *Nutrition and Cancer-An International Journal*, **23**, 233-246.

212. Peto, R., R. Doll, J.D. Buckley, M.B. Sporn (1981) Can dietary beta-carotene materially reduce human cancer rates? *Nature*, **290**, 201-208.

213. Pfitzner, I., P. I. Francz, H. K. Biesalski (2000) Carotenoid: methyl-β-cyclodextrin formulations. An improved method for supplementation of cultured cells. *Biochimica et Biophysica Acta*, **1474**, 163-168.

214. Platt, J.L. and K.A. Nath (1998) Heme oxygenase: Protective gene or Trojan horse? *Nature Medicine*, **4 (12)**, 1364-1365.

215. Ponnamperuma, R.M., Y. Shimizu, S.M. Kirchoff, L.M. De Luca (2000) Beta carotene pails to act as a tumor promoter, induces RAR expression and prevents carcinoma formation in a two stage model in male Sencar mice cells. *Nutrition and Cancer-An International Journal*, **37(1)**, 82-88.

216. Poswig, A., J. Wenk, P. Brenneisen, M. Wlaschek, C. Hommel, G. Quel, K. Faisst, J. Dissemond, K. Briviba, T. Krieg and K. Scharffetter-Kochanek (1999) Adaptive Antioxidant Response of Manganese-Superoxide Dismutase Following Repetitive UVA Irradiation. *Journal of Investigative Dermatology*, **112(1)**, 13 - 18.

217. Pourzand, C. and R.M. Tyrrell (1999) Apoptosis, the role of oxidative stress and the example of Solar UV Radiation. *Photochemistry and Photobiology*, **70(4)**, 380-390.

218. Pourzand, C., O. Reelfs, R.M. Tyrrell (2000) Approaches to define the involvement of Reactive Oxygen Species and Iron in Ultraviolet-A inducible Gene Expression. *Methods in Molecular Biology*, **99**, *Stress response: Methods and Protocols.*

219. Pourzand, C., R.D. Watkin, J.E. Brown and R.M. Tyrrell (1999) Ultraviolet A radiation induces immediate release of iron in human primary skin fibroblasts: the role of ferritin. *Proceedings of the National Academy of Sciences, USA*, **96**, 6751-6756.

220. Prakash, P., R.M. Russell, N.I. Krinsky (2001) In vitro inhibition of proliferation of estrogen-dependent and estrogen independent human breast cancer cells treated with carotenoids or retinoids. *Journal of Nutrition*, **131 (5)**, 1574-1580.

221. Radhakrishnan, A. and H.M. McConnell (2000) Electric field effect on cholesterol-phospholipid complexes. *Proceedings of the National Academy of Sciences, USA*, **97(3)**, 1073-1078.

222. Radhakrishnan, A., T.G. Anderson, and H.M. McConnell (2000) Condensed complexes, rafts, and the chemical activity of cholesterol in membranes. *Proceedings of the National Academy of Sciences, USA*, **97 (23)**, 12422-12427.

223. Rahman, I. and W. MacNee (2000) Regulation of Redox Glutathione Levels and Gene transcription in Lung Inflammation: Therapeutic approaches. *Free Radical Biology and Medicine*, **28(9)**, 1405-1420.

224. Raju, V.S. and M.D. Maines (1996) Renal ischemial reperfusion up-regulates heme oxygenase-1 (HSP32) expression and increases cGMP in rat heart. *Journal of Pharmacology and Experimental Therapeutics*, **277**, 1814–1822.

225. Raju, V.S., N. Imai and C.S. Liang (1999) Chamber-specific regulation of heme oxygenase-1 (heat shock protein 32) in right-sided congestive heart failure. *Journal of Molecular Cellular Cardiology*, **31**, 1581–1589.

226. Rao, A.V. and S Agarwal (1999) Role of lycopene as antioxidant carotenoid in the prevention of chronic diseases. *Nutrition Research*, **19(2)**, 305-323.

227. Rao, M.N., P. Ghosh and M. R. Lakshman (1997) Purification and Partial Characterization of a Cellular Carotenoid-binding Protein from Ferret Liver. *Journal of Biological Chemistry*, **272(39)**, 24455-24460.

228. Re, R., P.D. Fraser, M. Long, P.M. Bramley and C. Rice- Evans (2001) Isomerization of Lycopene in the Gastric Milieu. *Biochemical and Biophysical Research Communications*, **281**, 576-581.

229. Reddy, J.K. and T. Hashimoto (2001) Peroxisomal β-oxidation and Peroxisome Proliferator-Activated Receptor a: an adaptive metabolic system. *Annual Review of Nutrition*, **21**, 193-230.

230. Redmond, T.M., S. Gentleman, T. Duncan, S. Yu, B. Wiggert, E. Gantt, and F.X. Cuningham, Jr. (2000) Identification, expression and substrate specificity of a mammalian β-Carotene 15, 15'-Dioxygenase. *Journal of Biological Chemistry*, **276(9)**, 6560-6565.

231. Reeve, V.E., R.M. Tyrrell (1999) Heme oxygenase induction mediates the photoimmunoprotective activity of UVA radiation in the mouse. *Proceedings of the National Academy of Sciences, USA*, **96 (16)**, 9317-9321.

232. Ribaya-Mercado, J.D., F.S. Solon, M.A. Solon, M.A. Cabal-Barza, C.S. Perfecto, G. Tang, J.A.A. Solon, C.R. Fjeld and R.M. Russell (2000) Bioconversion of plant carotenoids to vitamin A in Filipino school-aged children varies inversely with vitamin A status. *American Journal of Clinical Nutrition*, **72**, 455-465.

233. Ribaya-Mercado, J.D., M. Garmyn, B.A. Gilcherst, R.M. Russell (1995) Skin lycopene is destroyed preferentially over β-carotene during ultraviolet irradiation in humans. *Journal of Nutrition*, **125**, 1854-9.

234. Ririe, K.M., R.P. Rassmussen and C.T. Witwert (1997) Product Differentiation by Analysis of DNA Melting Curves during the Polymerase Chain Reaction. *Analytical Biochemistry*, **245**, 154-160.

235. Rivier, M., I. Safonova, P. Lebrun, C.E.M. Griffiths, G. Ailhaud and S. Michel (1998) Differential Expression of Peroxisome Proliferator-Activated Receptor Subtypes During the Differentiation of Human Keratinocytes. *Journal of Investigative Dermatology*, **111**, 1116-1121.

236. Rock, C.L. (1997) Carotenoids: Biology and Treatment. *Pharmacology and Therapeutics*, **75**, 185-197.

237. Rock, C.L., J.L. Lovalvo, C. Emenhiser, M.T. Ruffin, S.W. Flatt, S.J. Schwartz (1998) Bioavailability of beta-carotene is lower in raw than in processed carrots and spinach in women. *Journal of Nutrition*, **128 (5)**, 913-916.

238. Rosenstein, B.S., J.M. Ducore (1983) Induction of DNA strand breaks in normal human-fibroblasts exposed to monochromatic ultraviolet and visible wavelengths in the 240-546 nm range. *Photochemistry and Photobiology*, **38 (1)**, 51-55.

239. Rossi, M.A., C. Di Mauro, M.U. Dianzani (2001) Experimental studies on the mechanism of phospholipase C activation by the lipid peroxidation products 4-hydroxynonenal and 2-nonenal. *International Journal of Tissue Reactions-Experimental and Clinical Aspects*, **23 (2)**, 45-50.

240. Roy, S., R. Luetterforst, A. Harding, A. Apolloni, M. Etheridge, E. Stang, B. Rolls, J.F. Hancock and R.G. Parton (1999) Dominant negative caveolin inhibits H-Ras function by disrupting cholesterol rich plasma membranes. *Nature Cell Biology*, **1**, 98-105.

241. Rucker, B.U., M. Haberle, H.U. Koch, P. Bocionek, K.H. Schriever, O.P. Hornstein (1991) Ultraviolet light hardening in polymorphus light eruption - a controlled study comparing different emission spectra. *Photodermatology and Photoimmunolology in Photomedicine*, **8**, 73 78.

242. Ryter, S.W. and R.M. Tyrrell (2000) The heme synthesis and degradation pathways: Role in oxidant sensitivity. Heme oxygenase has both prooxidant and antioxidant properties. *Free Radical Biology and Medicine*, **28(2)**, 289-309.

243. Ryter, S.W., R.M. Tyrrell (1998) Singlet molecular oxygen ($^1O_2$): A possible effector of eukaryotic gene expression. *Free Radical Biology and Medicine*, **24 (9)**, 1520-1534.

244. Sammut, I.A., R. Foresti, J.E. Clark, D.J. Exon, M.J. Vesely, P. Sarathchandra, C.J. Green and R. Motterlini (1998) Carbon monoxide is a major contributor to the regulation of vascular tone in aortas expressing high levels of haem oxygenase-1. *British Journal of Pharmacology*, **125**,1437–1444.

245. Sande, S., M.L. Privalsky (1996) Identification of TRACs (T-3 receptor-associating cofactors), a family of cofactors that associate with, and modulate the activity of, nuclear hormone receptors. *Molecular Endocrinology*, **10 (7)**, 813-825.

246. Scharffetter-Kochanek, K., M. Wlaschek, K. Briviba, H. Sies (1993) Singlet oxygen induces collagenase expression in human skin fibroblasts. *FEBS Letters*, **331**, 304 306.

247. Scheek, S., M.S. Brown, J.L. Goldstein (1997) Sphingomyelin depletion in cultured cells blocks proteolysis of sterol regulatory element binding proteins at site 1. *Proceedings of the National Academy of Sciences, USA*, **94**, 11179-83.

248. Schins, R.P.F., K. Donaldson (2000) Nuclear factor Kappa-B activation by particles and fibers. *Inhalation Toxicology*, **12**, 317-326 Suppl. 3.

249. Schlessinger, J. (2000) Cell signaling by receptors tyrosine kinases. *Cell*, **103**, 211-225.

250. Schoonjans, K., C. Brendel, D. Mangelsdorf, J. Auwerx (2000) Sterols and gene expression: control of affluence. *Biochimica et Biophysica Acta*, **1529**, 114-125.

251. Schoonjans, K., G. Martin, B. Staels, J. Auwerx (1997) *Current Opinion in Lipidology*, **8**, 159-166.

252. Scita, G, G.W. Aponte, G. Wolf (1992) Uptake and cleavage of beta-carotene by cultures of rat small intestinal-cells and human lung fibroblasts. *Journal of Nutritional Biochemistry*, **3 (3)**, 118-123.

253. Sen, C.L. and L. Parker (1996) Antioxidant and redox regulation of gene transcription. *FASEB Journal*, **10**, 709-720.

254. Shi, Y., M. Hon and R.M. Evans (2002) The peroxisome proliferator-activated receptor d, an integrator of transcriptional repression and nuclear receptor signaling. *Proceedings of the National Academy of Sciences, USA*, **99(5)**, 2613-2618.

255. Shindo, Y., E. Witt, D. Han and L. Packer (1994) Dose-response effects of acute ultraviolet irradiation on antioxidants and molecular markers of oxidation in murine epidermis. *Journal of Investigative Dermatology*, **104**, 470–475.

256. Silvers, A.L., G.T. Bowden (2002) UVA irradiation-induced activation of activator protein-1 is correlated with induced expression of AP-1 family members in the human keratinocyte cell line HaCaT. *Photochemistry and Photobiology*, **75 (3)**, 302-310.

257. Simons, K., E. Ikonen (1997) Functional rafts in cell membranes. *Nature*, **387**, 569-572.

258. Simons, K., D. Toomre (2000) Lipid rafts and signal transduction. *Nature Reviews Molecular Cell Biology*, **1**, 31-39.

259. Simons, K., E. Ikonen (2000) Cell biology - How cells handle cholesterol. *Science*, **290 (5497)**, 1721-1726.

260. Skoog, M.-L., K. Ollinger, M. Skogh. (1997) Microfluorometry using fluorescein diacetate reflects the integrity of the plasma membrane in UVA-irradiated cultured skin fibroblasts. *Photodermatology Photoimmunology and Photomedicine*, **13**, 37-42.

261. Soriani, M., C. Rice-Evans, and R.M. Tyrrell (1998) Modulation of the UVA activation of haem oxygenase, collagenase and cyclooxygenase gene expression by epigallocatechin in human skin cells. *FEBS Letters*, **439**, 253-257.

262. Soriani, M., P. Luscher, R.M. Tyrrell (1999) Direct and indirect modulation of ornithine decarboxylase and cyclooxygenase by UVB radiation in human skin cells. *Carcinogenesis*, **20 (4)**, 727-732.

263. Soriani, M., V. Hejmadi and R.M. Tyrrell (2000) Modulation of c-jun and c-fos transcription by UVB and UVA Radiations in Human Dermal Fibroblasts and KB Cells. *Photochemistry and Photobiology*, **71 (5)**, 551-558.

264. Stadtman, E.R. (1990) Metal ion-catalyzed oxidation of proteins - biochemical-mechanism and biological consequences. *Free Radical Biology and Medicine*, **9 (4)**, 315-325.

265. Stadtman, E.R. (1992) Protein oxidation and aging. *Science*, **257 (5074)**, 1220-1224.

266. Stahl, W. and H. Sies (2002) Carotenoids and protection against solar UV radiation. *Skin Pharmacology and Applied Skin Physiology*, **15 (5)**, 291-296.

267. Stahl, W. and H. Sies (1993) Physical quenching of Singlet Oxygen and cis-trans isomerization of carotenoids. *Annals of the New York Academy of Sciences*, **691**, 10-19.

268. Stahl, W. and H. Sies (1996) Lycopene: A Biologically Important carotenoid for humans. *Archives of Biochemistry and Biophysics*, **336(1)**, 1-9.

269. Stahl, W., J. Von Laar, H.-D. Martin, T. Emmerich, H. Sies (2000) Stimulation of gap junctional communication: comparison of acyclo-retinoic acid and lycopene. *Archives Biochemistry and Biophysics*, **373(1)**, 271-274.

270. Stahl, W., W. Schwarz, A.R. Sundquist, H. Sies (1992) Cis-trans isomers of lycopene and β-carotene in human serum and tissues. *Archives of Biochemistry and Biophysics*, **294(1)**, 173-177.

271. Stich, H.F., B.P. Dunn (1986) Relationship between cellular-levels of beta-carotene and sensitivity to genotoxic agents. *International Journal of Cancer*, **38 (5)**, 713-717.

272. Suematsu, M. and Y. Ishimura (2000) The heme oxygenase–carbon monoxide system: A regulator of hepatobiliary function. *Hepatology*, **31**, 3-6.

273. Sundaresan, M., Z.-X. Yu, V.J. Ferrans, D.J. Sulciner, J.S. Gutkind, K. Irani, P.J. Goldshmidt-Clermont and T. Finkel (1996) Regulation of reactive oxygen species generation in fibroblasts by Rac1. *Biochemical Journal*, **318**, 379-382.

274. Suruga, K., K. Mochizuki, R. Suzuki, T. Goda, S. Takase (1999) Regulation of cellular retinol-binding protein type II gene expression by arachidonic acid analogue and 9-cis retinoic acid in Caco-2 cells. *European Journal of Biochemistry*, **262 (1)**, 70-78.

275. Suschek, C.V., D. Bruch-Gerharz, H. Kleinert, U. Forstermann, V. Kolb-Bachofen (2001) Ultraviolet A (1) radiation induces nitric oxide synthase-2 expression in human skin endothelial cells in the absence of proinflammatory cytokines. *Journal of Investigative Dermatology*, **117 (5)**, 1200-1205.

276. Suttner, D.M., P.A. Dennery (1999) Reversal of HO-1 related cytoprotection with increased expression is due to reactive iron. *FASEB Journal*, **13 (13)**, 1800-1809.

277. Swinnen, J.V., W. Ulrix, W. Heyns and G. Verhoenen (1997) Coordinate regulation of lipogenic gene expression by androgens: Evidence for a cascade mechanism involving sterol regulatory element binding proteins. *Proceedings of the National Academy of Sciences, USA*, **94**, 12975-80.

278. Tabunoki, H., H. Sugiyama, Y. Tanaka, H. Fujii, Y. Banno, Z.E. Jouni, M. Kobayashi, R. Sato, H. Maekawa, K. Tsuchida (2002) Isolation, characterization, and cDNA sequence of a carotenoid binding protein from the silk gland of Bombyx mori larvae. *Journal of Biological Chemistry*, **277 (35)**, 32133-32140.

279. Takyi, E.E.K. (1999) Children's consumption of dark green, leafy vegetables with added fat enhances serum retinol. *Journal of Nutrition*, **129 (8)**, 1549-1554.

280. Termorshuizena, F., J. Garssena, M. Norvalc, L. Koulud, J. Laihiad, L. Leinod, C.T. Jansend, F. De Gruijlb, N.K. Gibbse, C. De Simonef and H. Van Loverena (2002) A review of studies on the effects of ultraviolet irradiation on the resistance to infections: evidence from rodent infection models and verification by experimental and observational human studies. *International Immunopharmacology*, **2(2-3)**, 263-275.

281. Thannickal, V.J. and B.L. Fanburg (2000) Reactive oxygen species in cell signaling. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, **279**, L: 1005-L1028.

282. The Alpha-Tocopherol, Beta-Carotene cancer prevention Study group (ATBC) (1994) The effect of β-carotene on the incidence of lung cancer and other cancers in male smokers. *New England Journal of Medicine*, **330**, 1029-35.

283. Tontonoz, P., E.D. Hu, B.M. Spiegelman (1994) Stimulation of adipogenesis in fibroblasts by PPAR-gamma-2, a lipid-activated transcription factor. *Cell*, **79 (7)**, 1147-1156.

284. Trekli, M., G. Riss, R. Goralczyk, R.M. Tyrrell (2003) Beta carotene suppresses UVA-induced HO-1 gene expression in cultured FEK4. *Free Radical Biology and Medicine*, **34(4)**, 456-464.

285. Tso, J.Y., X.H. Sun, T. Kao, K.S. Reece, R. Wu (1985) Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNA - genomic complexity and molecular evolution of the gene. *Nucleic Acids Research*, **13 (7)**, 2485-2502.

286. Tyrrell, R.M. (1991) UVA (320 - 380nm) Radiation as an oxidative stress, Oxidative stress: *Oxidants and Antioxidants*, Academic Press Ltd.

287. Tyrrell, R.M. (1994) The molecular and cellular pathology of Solar UV Radiation. *Molecular aspects of medicine*, **15 (1)**, 1-34.

288. Tyrrell, R.M. (1996) UV activation of mammalian stress proteins. *Stress inducible cellular responses*. Birkhauser Verlag Basel/Switzerland. Edited by U. Feige, R.I Morimoto, I. Yahara and B. Polla.

289. Tyrrell, R.M. (1996b) Activation of mammalian gene expression by the UV component of sunlight -from models to reality. *BioEssays*, **18(2)**, 139-148.

290. Tyrrell, R.M. (1997) Approaches to Define Pathways of Redox Regulation of an Eukaryotic Gene. *METHODS: A Companion to Methods in Enzymology*, **11**, 313-318.

291. Tyrrell, R.M. and M. Pidoux (1989) Singlet oxygen involvement in the inactivation of cultured human fibroblasts by UVA (334nm, 365nm) and near visible radiations *Photochemistry and Photobiology*, **49**, 407-412.

292. Tyrrell, R.M. and M. Pidoux (1986) Quantitative differences in host cell reactivation of ultraviolet damaged virus in human skin fibroblasts and epidermal keratinocytes cultured from the same foreskin biopsy. *Cancer Research*, **46**, 2665-2669.

293. Tyrrell, R.M. (1973) Induction of pyrimidine dimers in bacterial DNA by 365 nm radiation. *Photochemistry and Photobiology*, 17, 69-73.

294. Tyrrell, R.M. (1974) Induction of single strand breaks (alkali-labile bonds) in bacterial and phage DNA by near-UV (365 nm) radiation. *Photochemistry and Photobiology*, **20**, 395-399.

295. Tyrrell, R.M. (1984) Exposure of non dividing populations of primary human fibroblasts to UV (254nm) radiation induces a transient enhancement in capacity to repair potentially lethal cellular damage. *Proceedings of the National Academy of Sciences, USA*, **81**, 781-784.

296. Tyrrell, R.M. (1995) Biological dosimetry and action spectra. *Journal of Photochemistry and Photobiology B: Biology*, **31**, 35-41.

297. Tyrrell, R.M. (2000) Role for singlet oxygen in biological effects of ultraviolet A radiation. *Methods in Enzymology*, **319**, 290-296.

298. Tyrrell, R.M. and M. Pidoux (1987) Action Spectra for human skin cells. Estimates of the relative cytotoxicity of the middle UV, near UV, and violet regions of sunlight on epidermal keratinocytes. *Cancer Research*, **47**, 1825-29.

299. Tyrrell, R.M. and M. Pidoux (1988) Correlation between endogenous glutathione content and sensitivity of cultured human skin cells to radiation at defined wavelengths in the solar UV range. *Photochemistry and Photobiology*, **47**, 405-412.

300. Verma, A., D.J. Hirsch, C.E. Glatt, G.V. Ronnett, S.H. Snyder (1993) Carbon monoxide a putative neural messenger. *Science*, **259**, 381-384.

301. Vile, G.F. and R.M. Tyrrell (1995) UVA Radiation induced oxidative damage to lipids and proteins in vitro and human skin fibroblasts is dependent on iron and singlet oxygen. *Free Radical Biology and Medicine*, **18(4)**, 721-730.

302. Vile, G.F. and R.M. Tyrrell (1993) Oxidative stress resulting from UVA irradiation of human skin fibroblasts leads to a HO-dependent increase in ferritin. *Journal of Biological Chemistry*, **268(20)**, 14678-81.

303. Vogel, S., C.L. Mendelsohn, J.R. Mertz, R. Piantedosi, C. Waldburger, M.E. Gottesman, W.S. Blaner (2001) Characterization of a new member of the fatty acid-binding protein family that binds all-trans-retinol. *Journal of Biological Chemistry*, **276 (2)**, 1353-1360.

304. von Lintig, J. and K. Vogt (2000) Filling in the Gap in Vitamin A Research. *Journal of Biological Chemistry*, **275(16)**, 11915-20.

305. Wang, X.D., C. Liu, R.T. Bronson, D.E. Smith, N.I. Krinsky, R.M. Russell (1999b) Retinoid signaling and activator protein-1 expression in ferrets given beta-carotene supplements and exposed to tobacco smoke. *Journal of the National Cancer Institute*, **91 (1)**, 60-66.

306. Wang, X.D., M. Boudjelal, S. Kewon, J.J. Voorhes and G. Fisher (1999a) Ultraviolet irradiation of human skin causes functional vitamin A deficiency, preventable by all -trans retinoic acid pre-treatment. *Nature Medicine*, **5(4)**, 418-422.

307. Wang, X.-D., R. Russell, C. Liu, F. Stickel, D. Smith, N.I. Krinsky (1996) β-oxidation in Rabbit liver *in vitro* and in the perfused ferret liver contributes to

retinoic acid biosynthesis from β-apocarotenoic acids. *Journal of Biological Chemistry*, **271(43)**, 26490-98.

308. Wang, X.-D., G.-W. Tang, J. Fox, N.I. Krinsky and R.M Russell (1991) Enzymatic conversion of β-carotene into β-apocarotenals and retinoids by Human, Monkey, Ferret and Rat tissues. *Archives of Biochemistry and Biophysics*, **285(1)**, 8-16.

309. Wang, X.D., N.I Krinsky, P.N. Benotti, R.M. Russell (1994) Biosynthesis of 9-cis retinoic acid from 9-cis β-carotene in Human Intestinal Mucosa in Vitro. *Archives of Biochemistry and Biophysics*, **313(1)**, 150-155.

310. Wang, X.D., R. Russell (1999) Procarcinogenic and Anticarcinogenic Effects of β-carotene. *Nutrition Reviews*, **57(9)**, 263-272.

311. Wei, R.R, W.G. Wamer, L.A. Lambert and A. Kornhauser (1998) β-carotene uptake and effects on intracellular levels of retinol in Vitro. *Nutrition and Cancer*, **30(1)**, 53-58.

312. Wheeler, L.A., A. Aswad, M.J. Connor, N. Lowe (1986) Depletion of cutaneous glutathione and the induction of inflammation by 8-methoxypsoralen plus UVA radiation. *Journal of Investigative Dermatology,* **87 (5)**, 658-662.

313. White, J.A., B. Beckett-Jones, Y.-D. Guo, F.J. Dilworth, J. Bonasoro, G. Jones and M. Petkovich (1997) cDNA cloning of Retinoic Acid Metabolising Enzyme (hP450RAI) Identifies a novel family of cytochromes P450. *Journal of Biological Chemistry*, **272(30)**, 18538-18541.

314. Wilhelm, J., M. Hahn and A. Pingoud (2000) Influence of DNA target melting Behaviour on Real Time PCR quantification. *Clinical Chemistry*, **46(11)**, 1738-1743.

315. Williams, A.W., T.W.M. Boileau, S.K. Clinton, J.W. Erdman (2000) beta-carotene stability and uptake by prostate cancer cells are dependent on delivery vehicle. *Nutrition and Cancer-An International Journal*, **36 (2)**, 185-190.

316. Willoughby, D.A., A.R. Moore, P.R. Colville-Nash, D. Gillroy (2000) Resolution of inflammation. *International Journal of Immunopharmacology,* **22**, 1131-1135.

317. Wittwer, C.T., K.M. Ririe, R.V. Andrew, D.A. David, R.A. Gundry, U.J. Balis (1997) The LightCycler a microvolume multisample fluorimeter with rapid temperature control. *Biotechniques*, **22(1)**, 176-181.

318. Wlaschek, M., J. Wenk, P. Breneisen, K. Briviba, A. Schwarz, H. Sies, K. Scharffeter-Kochanek (1997) Singlet oxygen is an early intermediate in cytokine dependent UVA induction of interstitial collagenase in human dermal fibroblasts in vitro. *FEBS Letters*, **413**, 239-242.

319. Wolf, G. (2002) The effect of β-carotene on lung and skin carcinogenesis. *Carcinogenesis*, **23(8)**, 1263-1265.

320. Woodall, A.A., G. Britton and M.J. Jackson (1997b) Carotenoids and protection of phospholipids in solution or in liposomes against oxidation by peroxyl radicals: Relationship between carotenoid structure and protective ability. *Biochimica et Biophysica Acta*, **1336**, 575-586.

321. Woodall, A.A., S.W.M. Lee, R.J. Weesie, M.J. Jackson, G. Britton (1997a) Oxidation of carotenoids by free radicals: relationship between structure and reactivity. *Biochimica et Biophysica Acta: General Subjects*, **1336 (1)**, 33-42.

322. Woods, J.A., R.F. Bilton, and A.J. Young (1999) β-carotene enhances hydrogen peroxide induced DNA damage in human hepatocellular HepG2 cells. *FEBS Letters*, **440(2-3)**, 255-258.

323. Woutersen, R.A., A.P.M. Wolterbeek, M.J. Appel, H. van den Berg, R.A. Goldbohm, and V.J. Feron (1999) Safety Evaluation of Synthetic β-Carotene. *Critical Reviews in Toxicology*, **29(6)**, 515-542.

324. Wyss, A., G. Wirtz, W.-D. Woggon, R. Brugger, M. Wyss, A. Friedlein, H. Bachmann, W. Hunziker (2000) Cloning and expression of β,β-Carotene 15,15'-dioxygenase. *Biochemical and Biophysical Research Communications*, **271**, 334-336.

325. Yamamuchi, T., Y. Oike, J. Kamon, H. Waki, K. Komeda, A. Tsuchida, Y. Date, M.-X. Li, H. Miki, Y. Akanuma, R. Nagai, S. Kimura, T. Saheki, M. Nakazato, T. Naitoh, K. Yamamura, T. Kadowaki (2002) Increased Insulin sensitivity despite lipodystrophy in *Crebbp* heterozygous mice. *Nature Genetics*, **30**, 221-226.

326. Yan, W., G.-F. Jang, F. Haeseleer, Nesumi, J. Chang, M. Kerrigan, M. Campochiaro, P. Campochiaro, K. Palczewski and D.J. Zack (2001) Cloning and characterization of a human β,β-Carotene 15, 15'-Dioxygenase that is highly expressed in the retinal pigment epithelium. *Genomics*, **72**, 193-202.

327. Yeh, S.-L. and M.-L. Hu (2001) Induction of oxidative DNA damage in human foreskin fibroblast Hs68 cells by oxidized β-carotene and lycopene. *Free Radical Research*, **35**, 203-213.

328. Yoshida, T., P. Biro, T. Cohen, R.M. Muller, S. Shibahara (1988) Human heme oxygenase cDNA and induction of its mRNA by hemin. *European Journal of Biochemistry*, **171(3)**, 457-61.

329. Young, A.J. and G.M. Lowe (2001) Antioxidant and prooxidant properties of carotenoids. *Archives of Biochemistry and Biophysics*, **385(1)**, 20-27.

330. Young, A.R. (1997) Chromophores in human skin. *Physics in Medicine and Biology*, **42**, 789-802.

331. Young, A.R., J.M. Sheehan, C.A. Chadwick, C.S. Potten (2000) Protection by ultraviolet A and B sunscreens against in situ dipyrimidine photolesions in human epidermis is comparable to protection against sunburn. *Journal of Investigative Dermatology*, **115 (1)**, 37-41.

332. Zhang, H.K., J. Lehmann, B. Hoffmann, M.I. Dawson, J. Cameron, G. Graupner, T. Hermann, P. Tran and M. Pfahl (1992) Homodimer formation of Retinoid X-Receptor induced by 9-cis-retinoic acid. *Nature*, **358**, 587-591.

333. Zhou, X.F., X.Q. Shen, L. Shemshedini (1999) Ligand activated retinoic acid receptor inhibits AP-1 transactivation by disrupting c-Jun/c-Fos dimerization. *Molecular Endocrinology*, **13(2)**, 276-185.

334. Ziegler, R.G. (1989) A Review of Epidemiological Evidence that Carotenoids Reduce the risk of Cancer. *Journal of Nutrition*, **119**, 116-122.

335. Zijlstra, F.J., J.E. Vincent, W.M. Mol, H.C. Hoogsteden, P.T.W. Vanhal, R.C. Jongejan (1992) Eicosanoid levels in bronchoalveolar lavage fluid of young female smokers and nonsmokers. *European Journal of Clinical Investigation*, **22 (5)**, 301-306.

Appendix 1

A1. 1 Method for HPLC analysis of β-carotene and metabolites in cell culture medium samples and cell pellets (Georges Riss, Roche Vitamins Ltd)

Chemicals

All-trans-β-carotene, 9-cis-β-carotene, 13-cis-β-carotene, β-apo-4'-carotenal, β-apo-8'-carotenal, β-apo-12'-carotenal were from Roche Vitamins Ltd, Basel, Switzerland. Acetonitrile, tert-butylmethylether (t-BME), acetone and EtOH were from Merck, Darmstadt (Germany). Ammonium acetate, BHT, THF, and triethylamine for HPLC analysis were from Fluka Chemie AG, (Buchs, Switzerland). β-Cryptoxanthin pelargonate, the internal standard, was synthesized from β-cryptoxanthin and pelargonic acid chloride in a mixture of dichloromethane and pyridine according to reference [1]. HPLC grade solvents were additionally purified over basic aluminum oxide grade 1 (Camag, Muttenz, Switzerland).

Quantitative analysis of apo-carotenals, all-trans-β-carotene and cis-β-carotene in cultured cell lines and cell culture medium.

All procedures were performed under dimmed light and under cooled conditions. The HPLC solvent was degassed by sonication, and the samples were cooled at 6°C in the autosampler tray. Stock solutions of pure standard compounds i.e. β-carotene, its isomers, and apo-carotenals were prepared by dissolving 3-5 mg of the compound in n-hexane/dichloromethane 80:20 (v/v) containing 0.25 % BHT (v/w). Immediately afterwards, concentrations of suitable dilutions were determined by spectrophotometric measurements. Working and calibration solutions were prepared by evaporating required volumes of the stock solutions to dryness and re-dissolving the residue in the sample solvent mixture as described below. β–Cryptoxanthin pelargonate (1.0 nmol/ml) was used as an internal standard to correct possible injection losses. The cell culture medium (25 µl) was extracted by adding 225 µl of a solvent mixture EtOH/tBME/THF, 9:5:1 (v/v/v) containing the internal standard and 0.025% of BHT (v/w). The mixture was then vigorously vortexed for 1 minute and centrifuged for 3 minutes at 10 000 x g. Routinely, 25 µl of the clear supernatant were injected into the HPLC system.

The cell pellets were treated with 200µl acetone containing 0.025 % BHT (v/w) vortexed and dried in a speed-vac apparatus. The dried residue was redissolved in 200 µl of the solvent mixture described above, vortexed for 1 minute and centrifuged for 3 minutes at 10 000 x g. 25 µl or 50 µl of the clear supernatant were injected into the HPLC system. The chromatographic system consisted of a pump (L-6210), an autosampler (L-7200), a sensitive UV-VIS detector (L-7420) all from Merck-Hitachi, Ltd. (Tokyo, Japan) a solvent degasser unit (SDU 2003), and a column thermostat (Pelcooler), both from Labsource (Reinach, Switzerland).

HPLC analyses were performed isocratically on a C18-reversed phase column (Vydac 218TP54, 4x250 mm, The Separations Group, Hesperia, USA) with acetonitrile/tBME/aqueous ammonium acetate 80 mM/triethylamine,73:20:7:0.5, (v/v/v/v) as eluent. The flow rate was 1.5 ml/min, the peak detection was set at 450 nm. Chroma 2000 software from Bio-Tek Instruments (Basle, Switzerland) was used for system control, data acquisition and quantitative calculations.

References

1. Organikum; *Johann Ambrosius Barth Verlag Heidelberg - Leipzig,20. Auflage*; **454:**1996.

A1.2. Method for HPLC analysis of all trans- , 5-cis-and 13-cis- lycopene in cell culture medium and cell pellets (Georges Riss, Roche Vitamins Ltd.)

Chemicals

All-E-Lycopene, (5Z)-Lycopene and (13Z)-lycopene were from the chemical laboratories of Roche Vitamins AG (Kaiseraugst, Switzerland). Acetonitrile, 2-Propanol, Methanol and Ethanol pro analysis were from Merck (Darmstadt, Germany). *tert-* butylmethylether p.a., dichloromethane, Ammonium acetate p.a, butylated hydroxytoluene p.a., Tetrahydrofuran p.a., N,N-Diisopropylethylamine,p.a. were from Fluka Chemie AG (Buchs, Switzerland).

HPLC grade solvents for stock solution and dilution as well sample solvent mixtures were additionally purified over basic aluminum oxide grade 1 from Camag (Muttenz, Switzerland).

Quantitative analysis of all-E-, 5-cis- and 13-cis lycopene in cell culture medium and cell pellets.

Stock solutions of pure standard compounds *all-E*-Lycopene (5Z)- and (13Z)-lycopene were prepared by dissolving 3-5 mg of the compound in n-hexane/ dichloromethane 80:20 (v/v) containing 0.25 % of BHT (v/w). Immediately afterwards, concentrations of suitable dilutions were determined by spectrophotometric measurements, E (1%/1cm)= 3450 at 470 nm in hexane containing 2% dichloromethane.

Working and calibration solutions were prepared by evaporating, under nitrogen, required volumes of the stock solutions to dryness and re-dissolving the residue in the sample solvent mixture as described below

All extraction and analytical procedures were carried out in brown glass, and in a darkened room. Cell culture medium (25 µl) was extracted by adding 225 µl of a solvent mixture Ethanol/tBME/THF, 9:5:1 (v/v/v), containing 0.025% of BHT (v/w). The mixture was then vigorously vortexed for one minute, and centrifuged for 3 minutes at 10 000 x g. Routinely, 25 µl of the clear supernatant were injected into the HPLC system. The cell pellets were treated with 200 µl acetone containing 0.025 % BHT (v/w), vortexed and dried in a speed-vac apparatus. The dried residue was redissolved in 200 µl of the solvent mixture described above, vortexed for 1 minute and centrifuged for 3 minutes at 10 000 x g. Then, 25 µl or 50 µl of the clear supernatant were injected into the HPLC system. The chromatographic system consisted of a pump (System 520), an autosampler (565) a sensitive diode array detector (540+) all from Biotek-Instruments (Basel, Switzerland), as well as a solvent degasser unit (SDU 2003), and a column thermostat (Pelcooler) both from Labsource (Reinach, Switzerland).

HPLC analyses were performed isocratically on a C18-reversed phase column (Suplex-PKB-100 from Supelco (Bellafonte, PA, USA)) with acetonitrile / methanol / 2-propanol / aqueous ammonium acetate 2% / N,N-Diisopropylethylamine , 455:500:20:25:0.0.2 , (v/v/v/v) with 50 mg/L BHT, as eluent. The flow rate was 0.6 mL/min, and the peak detection was set at 470 nm. The solvent was degassed by sonication, and the samples were cooled at 7°C. Chroma 3000 software from Bio-Tek Instruments (Basel, Switzerland) was used for system control, data acquisition and quantitative calculations by the external standard method. Briefly, from the HPLC chromatograms of the standards, an average value of the relevant peak areas is divided by the corresponding spectrophotometrically measured concentration in a defined injection volume. This results in specific HPLC response factors (RF values) for each compound at defined chromatographic conditions.

Appendix 2

Comparison of different washing procedures to remove β-carotene from the outer surface of cells incubated with 3 µM β-carotene (Regina Goralczyk, Roche Vitamins Ltd.)

HaCat cells were incubated with 3µmol/L β-carotene for 3 days with daily medium changes (medium: FAD, + 2% NU serum). Medium was removed, and cells were washed either 3x or 6x with the various washing buffers. The cells were harvested for carotenoid HPLC analysis by trypsinisation followed by centrifugation. β-carotene was analysed in the cell pellets according to the established procedures.

Washing solution 3x washing	ß-carotene pmol/well	SD
PBS	32.9	6.7
PBS + BSA 2%	56.6	12.2
PBS + NU serum*	34.5	5.5
PBS + milkpowder	18.6	7.7
PBS + BSA/NU 1:1	46.5	17.9
PBS + BSA/milk powder 1:1	51.9	18.6
Washing solution 6X washing	ß-carotene pmol/well	SD
PBS	30.5	17.2
PBS + BSA 2%	16.5	2.3
PBS + NU serum*	47.3	16.2
PBS + milkpowder	57.6	20.2
PBS + BSA/NU 1:1	72.7	19.5
PBS + BSA/milk powder 1:1	66.2	18.0

n=6

- NU serum = commercial semisynthetic medium containing 15% fetal calf serum

The best washing effect was observed, when cells were washed 6 x with PBS 2% BSA. This reduced the adherent β-carotene at most, with the lowest variation.